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ENVIRONMENTAL PROTECTION AGENCY
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SUBSTITUTE CHEMICAL PROGRAM - THE FIRST YEAR OF PROGRESS

PROCEEDINGS OF A SYMPOSIUM

Volume II

Toxicological Methods and Genetic Effects Workshop

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY

SHERATON-FREDERICKSBURG MOTOR INN
FREDERICKSBURG, VIRGINIA

JULY 30 - AUGUST 1, 1975

The Proceedings of the SUBSTITUTE CHEMICAL PROGRAM -- THE FIRST YEAR OF PROGRESS are published in four volumes. Volume I contains speeches and discussion from the Plenary Session, the agenda, and lists of participants and speakers. Volumes II, III, and IV cover the Toxicological Methods and Genetic Effects Workshop, the Ecosystems/ Modeling Workshop, and the Chemical Methods Workshop, respectively.

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TOXICOLOGICAL METHODS AND GENETIC EFFECTS
RESEARCH PROGRESS SESSION
INTRODUCTION

Dr. August Curley*
and
Dr. Lamar B. Dale, Jr.**

The Research and Special Studies as outlined in this progress section are being conducted cooperatively under the Substitute Chemical Program by the Office of Research and Development and the Office of Pesticide Programs, Criteria and Evaluation Division. These studies are conducted as:

1. An intramural research effort by the Office of Research and Development, Environmental Research Center, Health Effects Research Laboratory, Environmental Toxicology Division, Research Triangle Park, North Carolina (formerly the National Environmental Research Center, Pesticides and Toxic Substances Effects Laboratory);
2. As an extramural contractual research effort with the several individual laboratories as indicated in the program; and
3. Through an interagency research program at the National Center for Toxicological Research, Jefferson, Arkansas, jointly sponsored by FDA and EPA.

The major areas of research included in this scope of the cooperative Substitute Chemical Program are:

1. Basic short- and long-term toxic effects research which includes methodology development and evaluations; and
2. In vivo and in vitro screening tests to address the problems in carcinogenesis, mutagenesis, and teratogenesis associated with possible human exposure to these compounds.

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*Office of Research and Development, Environmental Research Center, Health Effects Research Laboratory, Environmental Toxicology Division, Research Triangle Park

**Office of Pesticide Programs, Criteria and Evaluation Division, EPA

This research and special studies effort is the primary responsibility of the Office of Research and Development and supports the internal review process of the Substitute Chemical Program, including the Cooperative Liaison Studies effort between other Governmental agencies, pesticide manufacturers, and academia as accomplished through integrated planning by the Office of Pesticide Programs/Criteria and Evaluation Division and Office of Research and Development. This research effort responds to specific deficiencies in the scientific data base as identified by the review process. Through these reviews, new toxicity data are generated where required; testing, screening techniques, and protocols are developed to evaluate certain toxicological aspects of potential substitutes; and behavioral, pulmonary-hepato metabolic alterations, including hormonal effects, are determined.

Through the implementation of these studies, the Substitute Chemical Program expects to determine the suitability of these chemicals which now or in the future may serve as replacements for those major or minor use pesticides that are currently under internal review, or those which have been suspended, granted limited uses, cancelled, or are in litigation to be suspended or cancelled. In short, the overall intent of this program is to assess the human health hazard associated with the use of these compounds in pest control.

This session, which is called the Toxicological Review Session, has been divided into several subsessions: namely, Inhalation Studies; Acute, Subacute and Chronic Studies; Epidemiological Residue Studies; Carcinogenesis, Mutagenesis, and Teratogenesis Studies. This afternoon we will have research progress reports on the inhalation studies and the acute, subacute, and chronic studies. Tomorrow Dr. Cranmer, Director of the National Center for Toxicological Research at Jefferson, Arkansas, will chair the session on carcinogenesis, mutagenesis, and teratogenesis testing. The evening session, which is a continuation of the studies in carcinogenesis, mutagenesis, and teratogenesis, will be chaired by Ms. Jean Pulliam. We will follow the program as outlined.

INHALATION TOXICOLOGY OF SUBSTITUTE CHEMICALS

Dr. James T. Stevens*

NOT AVAILABLE

FOR

PUBLICATION

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*National Environmental Research Center, Research Triangle Park, North Carolina

INHALATION TOXICOLOGY

Dr. Gordon W. Newell*

A thorough investigation of insecticides is being conducted by EPA with a view to determining their effects on health and the environment. From a review of the literature and laboratory studies, it is anticipated that alternate chemicals for pest control might be recommended for primary use in place of others currently used.

To support these aims the Toxicology Department at Stanford Research Institute was commissioned by EPA to investigate the acute toxicity of five organophosphate insecticides in experimental animals. The modes of application included the intragastric, intravenous, percutaneous, and inhalation routes. Of necessity, this program was divided into five phases: 1) generation of respirable particles; 2) analytical methods to determine the aerosol concentration in the exposure chamber; 3) acute toxicity study in rats; 4) gross and histopathological changes of animal organs; and 5) the analysis of animal tissue for insecticide content.

The compounds studied in this program were technical-grade pesticides: Azodrin, Guthion, methyl parathion, parathion, and Thimet.

Phase 1 - Generation of Respirable Particles

This phase had as its primary aim the generation of a reliable and reproducible aerosol of the test pesticides. In order to obtain the maximum biological effects, the mass mean particle size was to be in the range of 0.5 to 3 microns, and constitute approximately 90 percent of the particulates. Of the remainder, those particles less than 0.5 micron and larger than 4.0 microns should be less than 10 percent. Only under these conditions could maximum lung penetration be achieved.

These aerosols can be generated most effectively by either pneumatic or ultrasonic means. Both techniques have been used in this study.

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*Department of Toxicology, Stanford Research Institute

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Concentrations of the insecticides were to be maximized at 200 mg/m^3 and any desired ratio dilutions were to be controllable through the use of critical flow orifices and the regulation of air pressure in the upstream manifold.

Both the insecticide concentration and the particle size distribution were to be monitored. Insecticide concentration is discussed later, while particle size distribution has been monitored by one of three techniques, depending on the vapor pressure of the candidate: 1) by optical means -- particles captured on a non-wetting surface are photographed and counted with a minimum time delay; 2) by physical means -- surface impaction; and 3) electronically -- using a cryogenic stage within a scanning electron microscope.

With the SRI exposure chambers it is possible to present the test animals, radially situated, to a down draft of the aerosol, in such a way that all 10 animals breathe air within a 16-square-inch area. By so doing, variations in aerosol density due to abnormal turbulent deposits within the air stream are minimized.

Description of Aerosol Exposure Facilities

General Description

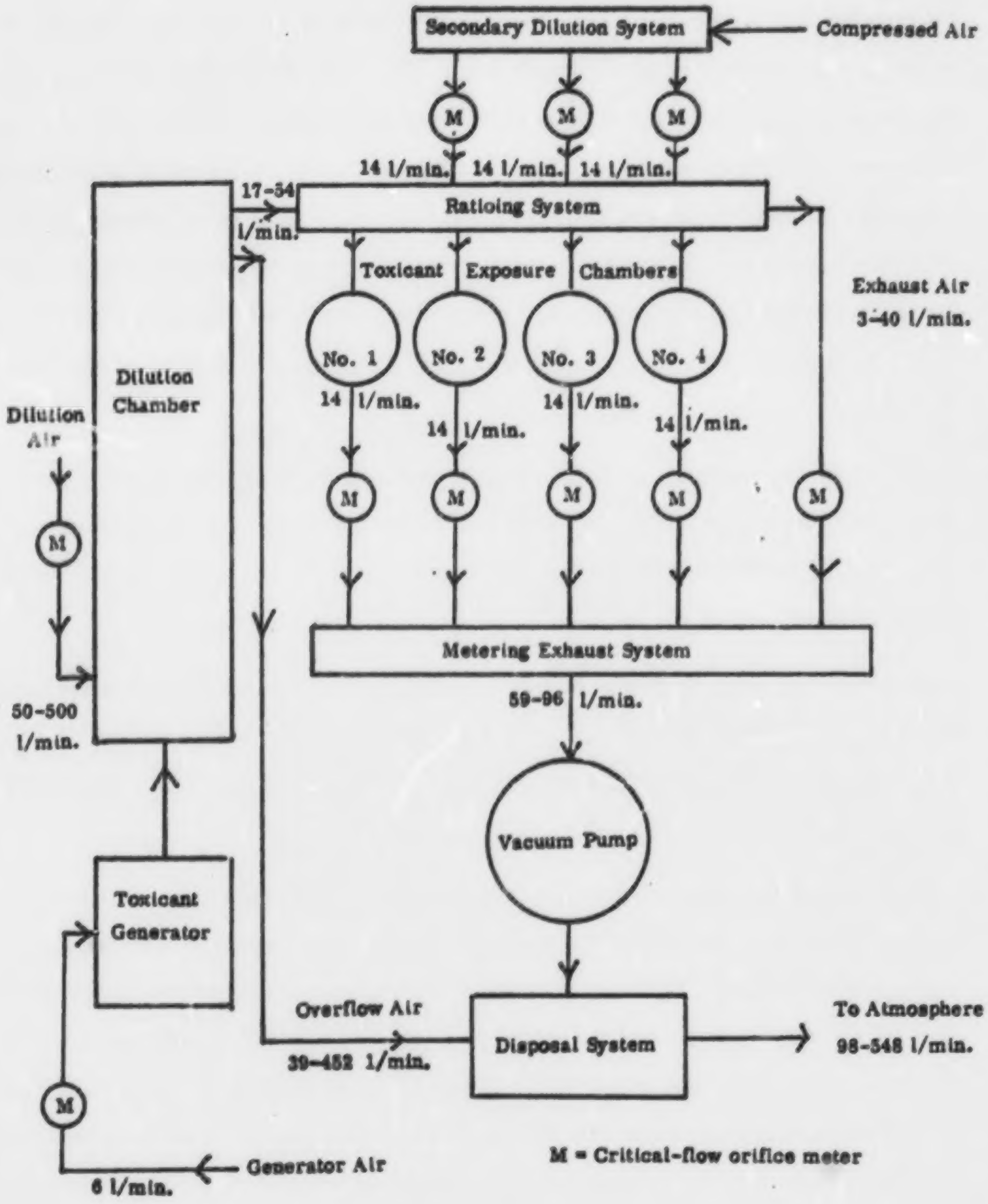
The aerosol exposure system provides for the simultaneous exposure of groups of animals to successively lower concentrations of a test material. It permits predetermined and independent control of the concentration in the first chamber and the ratio of concentrations in successive chambers. The system is composed of five component elements: 1) a generator, 2) a primary dilution chamber, 3) a concentration ratioing system, 4) the exposure chambers, and 5) a disposal system. The system is shown in Figure 1.

Initial Dilution

The pesticide is introduced to the dilution chamber in vapor or aerosol form by an ultrasonic or pneumatic generator. The aerosol concentration in the first chamber (desired maximum exposure concentration) is controlled by the amount of dilution air metered into the dilution chamber.

Figure 1

Condensed Schematic Diagram of Toxicant Exposure System


Secondary Dilution System
Compressed Air
M
14 l/min.
14 l/min.
14 l/min.
17-54 l/min.
Ratioing System
Toxicant Exposure Chambers
No. 1
No. 2
No. 3
No. 4
14 l/min.
Exhaust Air
3-40 l/min.
Dilution Chamber
Dilution Air
Metering Exhaust System
59-96 l/min.
50-500 l/min.
Vacuum Pump
Toxicant Generator
Overflow Air
39-452 l/min.
Disposal System
To Atmosphere
98-548 l/min.
Generator Air
6 l/min.
M = Critical-flow orifice meter

Some of the diluted stream goes to the ratioing system where a portion is drawn off into the first exposure chamber. The balance of the stream entering the ratioing system is successively diluted with clean air. A portion of each diluted stream is passed to an exposure chamber before the next dilution. Thus, each chamber receives a successively lower toxicant concentration. The ratio of concentration between successive chambers is determined by the flow rate leaving the ratioing system relative to that entering and leaving the ratioing system. Thus, the ratio R can be changed by simply changing the exhaust air rate from the ratioing system, which is actually done by changing the size of the orifice used to meter the exhaust air rate.

All flows are metered by critical flow orifices. Secondary dilution rates are metered by flow from a compressed air main to essentially atmospheric pressure. Chamber flows and the ratioing system exhaust are metered by flow from substantially atmospheric pressure to high vacuum ($<$ 1/3 atmosphere).

Although the dilution ratio is held constant, a different ratio between the chambers could be established by a corresponding selection of the respective orifice plates used for the secondary dilution air supply metering and for metering the flow through the test chambers.

Any of the gas from the dilution chamber that is not required (or demanded by) the ratioing system overflows directly to the disposal system through a filter. The exhaust from the ratioing system vacuum pump also goes to the disposal system. No general filter is used on the ratioing system, since each of the ratioing system streams has its own filter. These filters remove the aerosol. Any aerosol particles escaping the filter or any vapors are destroyed by passing them through a gas-fired incinerator. The gas rate is set to adjust the outlet incinerator temperature from 1400 to 1500° F. The total gas residence time in the incinerator is in the order of one second.

All the gases from the entire system are exhausted to the atmosphere by means of a compressed-air operated ejector located downstream of the incinerator. There is also a provision for cooling the gases leaving the incinerator by induced room air.

Safety Features

System pressure is controlled by adjusting the compressed air rate to the exhaust ejector. The absolute value of this pressure is not critical to the operation of the ratioing system. The pressure is maintained at an arbitrary -0.5 inch water to avoid leakage of toxicants into the room. A warning system has also been provided to give an alarm if the system pressure exceeds -0.2 inch water. This alarm consists of both a flashing red light and a buzzer. The buzzer can be deactivated by a switch.

A heating element is provided in the dilution air feed line, and is provided to compensate for any heat requirements for evaporating residual solvents from the aerosol particles emanating from the generator.

Humidity of the dilution air is controlled by saturating a predetermined portion of the primary dilution air with a dual primary dilution air metering system, an air-water contacting system, and a cyclone entrainment separator.

The valving in the test system is so arranged that any one test chamber may be sampled or disconnected from service during a run without influencing conditions at any other test chamber.

Critical aspects that have been considered in the design are 1) versatility, convenience, and reliability consistent with reasonable cost; 2) safety of the operator; 3) thoroughness of mixing of air streams; 4) avoidance of deposition of aerosols in the apparatus; 5) ease of cleaning; 6) short equilibration time (probably less than one minute); and 7) visibility of animals. The dimensions of the various components have been based on a detailed quantitative evaluation of all these factors. In many cases a compromise is necessary. This is particularly true in the case of aerosols, where thoroughness and rapidity of mixing are not compatible with avoidance of aerosol deposition on the walls. The ducts and mixing elements have been designed to achieve what is considered adequate mixing with an acceptable minimal amount of wall deposition. From test results, it can be inferred that mixing is at least 95 percent complete and that aerosol deposition is well under 5 percent.

The animal test chambers have been designed to achieve a reasonably uniform aerosol flow across all animals with a minimum opportunity for loss of aerosol particles. The animals are confined by wire enclosures so that they are almost immobile with their noses exposed into the incoming aerosol stream. There is room for 10 rats per chamber and the ring through which the aerosol flows into which the rats' noses extend is 5.5 inches in diameter. The total volume of the animal cages, including the stainless steel approach and discharge cones, is approximately 30 liters. All animals are visible from the outside at all times, since the top of the chamber is transparent. At a chamber air flow of 14 liters per minute, the air velocity past the rats' noses averages 1.5 cm/sec.

Particle size measurements were made of the various pesticides using the Royco Optical particle counter in conjunction with a dilution system, the development of which was sponsored by SRI. Without the diluting system, the aerosol concentration would have been too high (even from the last animal chamber) for the Royco to handle. The data points are based on the actual count obtained with samples taken from the last animal chamber and diluted to various extents before reaching the Royco counter. The average curve through these data gives the size distribution of the aerosol on a number basis. The mass distribution curve was calculated from this average number distribution curve. With the exception of Guthion (which gives a somewhat finer aerosol), all the other materials (Thimet, ethyl parathion, and methyl parathion) were sprayed in neat form and gave essentially the same size distribution. Because Guthion was sprayed as a 20 percent solution in DMSO, it is to be expected that the final average aerosol particle size should be about $(0.2)^{1/3}$ or 0.6 that of the other pesticides.

Phase 2 - Sampling of Chamber Contents

Chamber contents are sampled by taking grab samples of the material during the animals' exposure period. The sampling train consists of a pump pulling air at approximately one liter per minute through a particulate filter and a solvent trap. The length of time for sampling depends on both the compound and its concentration in the chamber.

Studies were conducted to determine that the aerosol concentration did not vary with time or location within any given chamber, and also to verify that serial concentration between chambers was within the hypothetical norm.

Phase 3 - Chemical Analysis of Chamber Contents

A gas chromatographic (GC) method for analyzing the pesticides was developed using a flame photometric detector (FPD). The FPD detector was modified as suggested by Burgett *et al.* (J. Chromatog. Sci. 12: 356, 1974) to eliminate solvent flameout and to increase response linearity. For Azodrin, analyses were performed under the following conditions:

- Column, 3 percent SE-20 (2 mm x 4') on 80/100 mesh Gas-Chrom Q
- Temperature, 170°
- Flow rate, 60 ml/min. N_2

Quantitation was based on the internal standard method with respect to methyl parathion. The minimum detectable limit for Azodrin was found to be 20 ng.

Direct air sampling (5 ml) of the Chamber S (highest concentration) was unsuccessful. Therefore, preweighed nucleopore filters were weighed after a timed exposure to the aerosols. The filters were extracted with 1 ml of acetone, and methyl parathion as an internal std. was added.

Phase 4 - Acute Toxicity

Acute toxicity studies were conducted with parathion, methyl parathion, Thimet, Azodrin, and Guthion, administered by four routes. At least four effective insecticide concentrations were used in each case.

Table 1

Administration Route	Vehicle	Maximum Dosage
Oral	Water or propylene glycol	5000 mg/kg
Dermal	Water or propylene glycol	2000 mg/kg
Systemic	Ethanol, propylene glycol	2000 mg/kg
Inhalation	DMSO	200 mg/kg

All studies are carried out on Sprague-Dawley rats weighing between 220 to 250 grams. All animals are fasted 24 hours prior to dosing, returned to their cages after exposure, and watched closely for pharmacotoxic signs. All rats are held for 14 days to determine the presence or absence of late deaths. Body weights are determined at the 1- and 2-week levels. Food and water are freely available after dosing. Animals that die during the holding period are necropsied. and sacrificed animals are also necropsied. Tissues are gathered at necropsy from all animals exposed to the two highest concentrations of insecticide, regardless of route.

Acute Oral Toxicity: The four dosage levels are calculated on the fasted weights of the animals. A glass syringe fitted with a ball tip needle is used for administration. After dosing, the animals are returned to the cage for observation.

Percutaneous Toxicity: Twenty-four hours before dosing, rats have their backs and sides clipped free of hair and are returned to their cage. Dosage of insecticide is based on the fasted weight and is applied to the backs and sides in a suitable vehicle; runoff is not tolerated. While dosages vary, the volume added to each animal is constant. A treated animal is wrapped in an impervious layer and placed in a restrainer for 1 or 4 hours. Following this exposure, surplus material is wiped off using cotton moistened with the diluent vehicle and the rat is returned to its cage.

Acute Systemic Toxicity: Fasted rats are placed in a restrainer and dosed intravenously through the dilated tail vein. The dosage rate varies, but the total administered volume remains constant. Treated animals are returned to their cages for observation.

Acute Inhalation Toxicity: Fasted rats of known weight are restrained in individual cells, with only the anterior portion of the head exposed. These cells are placed in the exposure chamber where the animals receive a 1- or 4-hour exposure to the aerosolized insecticide. These rats are returned to their cages with minimal cleaning and held for observation.

Phase 5 - Gross and Microscopic Pathology

Necropsies are conducted on those rats dosed at insecticide levels which are used in developing the LD_{50}.

Histopathological assessments are routinely carried out on the lungs of rats exposed to the high concentrations of insecticides administered via the inhalation route. Additional histopathological investigation is performed on all tissues where a repetitive gross abnormality has been detected.

Phase 6 - Gross and Histopathological Changes

No important gross changes have been observed. Approximately 320 H&E slides (from 76 or 78 rats) of liver, kidney, lung, brain, and skin were examined microscopically. Lesions in animals in the various treatment groups were recorded and compared. Since the sizes of the groups were often inadequate and some groups were not represented, our conclusions are tentative.

Table 2: Number of Sampled Tissues

	Route of Administration			
	Oral	IV	Dermal	Total
Guthion	8	2	0	10
Parathion	4	0	7	11
Methyl parathion	0	16	9	25
Thimet	0	7	5	12
Azodrin	8	6	5	19
Total	20	31	26	77

Moreover, most lesions were of a type that is usual and spontaneous and, hence, unlikely to be related to treatment. Nonetheless, the overall incidence of lung lesions in rats treated with Azodrin intravenously and orally was approximately half that in rats treated with other pesticides by the oral, intravenous, or dermal routes.

Lung tissues are being taken and examined from animals undergoing various inhalation exposures, but we do not believe continuation of histopathologic evaluation of any of the other tissues (previously examined) is worthwhile.

We have seen no brain lesions, some degree of congestion in many or most livers and kidneys, and a variety of common spontaneous lesions in lungs. No lesion resulting from treatment or exposures has been recognized.

Chemical Analysis of Tissues for Insecticide Content: Body Burden vs. Exposure

The method of Vukovich et al. (J. Ag. & Food Chem. 17: 1190, 1969) was the procedure initially used for the cleanup and analysis of these organophosphates in tissues. Initially, a Varian Aerograph 1800 gas chromatograph with an electron capture detector was applied. Subsequently, a Tracor Model 222 gas chromatograph, equipped with a flame photometric detector in conjunction with a 5 percent OV-17 column 43" in length and 3/16" I.D. was found to be more sensitive and was used in all subsequent analyses. For example, whole blood was spiked with 0.01 ppb of methyl parathion and yielded a recovery in plasma of 95-106 percent. For parathion recovery was 88-104 percent.

For the basic extraction and cleanup procedure, 1 ml of plasma is mixed with 20 μl of conc. HCl and 1.5 ml of N-hexane, shaken for 20 minutes on a mechanical shaker, centrifuged for 10 minutes to break the emulsion, and an appropriate aliquot injected into the GC for measurement.

Improved recovery was accomplished for parathion and methyl parathion with the use of a Florisil column, The column, half an inch in width, is packed with Florisil to a depth of 10 cm, to which 1 inch of anhydrous sodium sulfate is added. Florisil is preconditioned for at least 24 hours at 130^{o} C and then allowed to cool to room temperature before making up the column. The column is rinsed with 10 ml of hexane and then the sample in hexane is added. Elution is done with 15 ml of 10 percent diethyl ether in hexane, followed by an equal volume of 50 percent ether in hexane. The eluants are concentrated on a steam bath and final evaporation to 0.5 - 1.0 ml is done with a gentle stream of nitrogen. The sample is then injected into the GC. By this technique recoveries of 95 percent methyl parathion, 104 percent of parathion, and 98 percent of Thimet were attained. For elution of Guthion from the Florisil column, the technique of Zweig and Sherma in using $CHCl_3$ as the eluant was found to be a satisfactory procedure, with recoveries of 94 percent obtained.

Reproducibility of results made from aliquots of the same sample, starting from the initial Vukovich extraction step, were excellent. Plasma samples obtained from rats 1 hour after a 155-minute inhalation exposure to methyl parathion at 152 mg/m^3 had the following duplicate values.

4A	26 and 23 ppb
4B	18 and 15 ppb
4C	26 and 27 ppb
4D	25 and 24 ppb

Despite the good sensitivity and reproducibility of the analytical methods, there was great variation in the plasma values between individual rats which received the same pesticide treatment, regardless of the route of administration. Plasma concentrations of Thimet in 7 rats given a 1.6 mg/kg intravenous dose 30 minutes after treatment ranged from 6 to 30 ppb; a 2.0 mg/kg intravenous dose after 30 minutes was in the range of 7 to 49 ppb; a 2.5 mg/kg dose showed values ranging from 12 to 46 ppb. A dermal application of 50 mg/kg of parathion after 2 hours showed plasma concentration ranging from 38 to 80 ppb; after 4 hours the values in other rats ranged from 40 to 79 ppb; at 6 hours 76 to 102 ppb; and at 24 hours 35 to 56 ppb. Similar variations were seen for Thimet given intravenously and for parathion given intravenously.

In view of these major variations, it was apparent that body burden could not be reliably assessed by chemical measurements of blood plasma. Thus, we are now measuring whole blood cholinesterase inhibition by the Hestrin method as modified by Fleisher and Pope. Initial results show greater uniformity of response between animals subjected to identical treatment.

Table 3 will give you a few summarizations of data. These are rounded values, of course, for easy comparison. We tried to put together values by the various procedures for the five compounds. The percutaneous LD_{50} value -- and these are for male rats -- for Guthion was not available by the time we made the slide. It is approximately 210 to 200 mg/kg, in that range.

Table 3: Organophosphate Pesticide LD_{50} and LC_{50} Values in Male Rats

	LD_{50}(mg/kg)			LC_{50}(mg/m^3)
	Oral	Intravenous	Percutaneous	Inhalation
Parathion	14	6	49	530
Methyl Parathion	12	9	110	257
Thimet	4	2	9	63
Azodrin	35	12	220	178
Guthion	16	18		69

And, as you might expect, parathion and Thimet show the highest degrees of toxicity across the various routes of treatment. Table 4 shows the same types of data for female rats. Again, there are similar types of responses. Parathion and Thimet in the female rat show increased sensitivity or they produce a higher level of toxicity, but this is not consistent for the other three compounds.

Table 4: Organophosphate Pesticide LD_{50} and LC_{50} Values in Female Rats

	LD_{50}(mg/kg)			LC_{50}(mg/m^3)
	Oral	Intravenous	Percutaneous	Inhalation
Parathion	8	4	20	200±40
Methyl Parathion	18	15	120	423
Thimet	1	1	4	15
Azodrin	20	9	206	179
Guthion	18	8	222	50-60

We have attempted to interrelate the kinds of data from one type of exposure to another. In Table 5 we used Guton's formula to convert body weight to respiratory rate. This formula uses 2.1 grams of body weight to the three-fourths power, and obtains the breathing rate in milliliters per minute. We've used the table as an approach for this conversion.

Table 5: Conversion

$$(\mathrm{LD}_{50})\,\mathrm{mg/kg} \rightleftarrows \mathrm{mg/m}^3\,(\mathrm{LC}_{50})$$

$$\frac{\mathrm{mg/kg}}{\text{No. of rats/kg}} = \mathrm{Ymg/rat} = \mathrm{Ymg}/6\,\mathrm{l}\ *$$

$$\frac{167 \times \mathrm{Ymg}}{167 \times 6\,\mathrm{l}} = \frac{167\ \mathrm{mg}}{1000\ \mathrm{l}} = \frac{167\ \mathrm{Ymg}}{\mathrm{m}^3}$$

*Air breathed by a 200-g rat in 1 hour; 100 breaths per minute and 1 ml per breath.

If one knows that a value has an LD_{50} of so many milligrams per kilogram, and knowing the number and weight of rats used, you can determine the milligrams per rat, which is equivalent to the milligrams of compound Y per 6 liters of air. The 6 liters is the amount of air breathed by a 200-gram rat in one hour, assuming 100 breaths per minute, and one milliliter volume per breath.

Therefore, if we convert the 6-liter volume to a cubic meter, or 1,000 liters, you multiply upper and lower cases of the formula by 167. We used this approach in the next several tables. If these data were idealized as, for example, having an LD_{50} of five milligrams per kilo, this would be equivalent to producing an LC_{50} of 167 milligrams per cubic meter for a 1-hour exposure, or 42 milligrams per cubic meter for a 4-hour exposure (Table 6).

Table 6: Conversion Data

$mg/kg \rightleftarrows mg/m^3$

(Assume 200-g rat breathes 100 ml/min.)

mg/kg	1 Hour mg/m^3	4 Hours mg/m^3
1	33	8
2	67	17
3	100	25
4	133	33
5	167	42
7.5	250	62.5
10	334	83
20	668	167

Now, with the data that we have obtained, we attempted to make some comparisons. Using the intravenous route as our basis of comparison against the LC_{50} inhalation values, we attempted to develop some factors, at least for the organophosphates. For the male rats, by these two comparisons, we have a ratio of approximately 0.9 to 2.6, or an average of 1.4 (Table 7).

Table 7: LD_{50} Comparisons (g/kg) - Male Rats

	(Base) Intravenous	F_3	Inhalation
Parathion	6	2 2/3	16
Methyl Parathion	9	0.85	7.7
Thimet	2	1	2
Azodrin	12		
Guthion	7.5	1.2	8.5
Average		1.4	

For the female rats, the ratios are a little tighter, an average of about 0.9 (Table 8). In comparing the intravenous values against oral values, the range factors are greater, ranging from 1.3 to 3.4, and averaging at 2.1 (Table 9). In the same comparison for female rats, the values are a little tighter, with an average of 1.7 (Table 10).

Table 8: LD_{50} Comparisons (g/kg) - Female Rats

	(Base) Intravenous	F_3	Inhalation
Parathion	4.5	1.5	6±1
Methyl Parathion	15	0.85	12.7
Thimet	1.2	0.4	0.5
Azodrin	9	0.6	5.5
Guthion	7.5	0.9	6-7.4
Average		0.85	

Table 9: LD_{50} Comparisons (g/kg) - Male Rats

	(Base) Intravenous	F_3	Oral
Parathion	6	2 1/3	14
Methyl Parathion	9	1 1/3	12
Thimet	2	2	4
Azodrin	12	3.4	3.5
Guthion	7.5	2	15.5
Average		2.1	

Table 10: LD_{50} Comparisons (g/kg) - Female Rats

	(Base) Intravenous	F_1	Oral
Parathion	4.5	1.8	8
Methyl Parathion	15	1.2	18
Thimet	1.2	1.2	1.4
Azodrin	9	2.1	19
Guthion	7.5	2.4	18
Average		1.7	

In attempting to compare intravenous against percutaneous, the ratios run all over the map (Tables 11 and 12). There is no basis for even a crude factor for comparison for both male and female rats.

Table 11: LD_{50} Comparisons (g/kg) - Male Rats

	(Base) Intravenous	F_2	Percutaneous
Parathion	6	8	49
Methyl Parathion	9	12	110
Thimet	2	4.5	9
Azodrin	12	18	220
Guthion	7.5	60	455
Average		None	

Table 12: LD_{50} Comparisons (g/kg) - Female Rats

	(Base) Intravenous	F_2	Percutaneous
Parathion	4.5	4.5	19.5
Methyl Parathion	15	8	120
Thimet	1.2	3 1/4	3.9
Azodrin	9	23	206
Guthion	7.5	30	220
Average		None	

There are certain differences between intravenous and inhalation responses, assuming that the intravenous approach is the most direct and the least likely to have complicating interferences. The dose is gradually acquired by the inhalation route. During an extended inhalation exposure, detoxification, metabolism, elimination of the compound from the body, storage in tissues, etc., can occur. All of these variables can affect the ratioing between inhalation and intravenous comparisons.

Of course, if there is lung damage, gaseous exchange can be affected. Thus, toxic effects may increase or decrease due to lung passage; absorption may be incomplete via the lungs, and pulmonary excretion may not occur (Table 13). Tentative conclusions from those data suggest that the oral LD_{50} is approximately two times the intravenous LD_{50} dose. For inhalation, compared to intravenous, it is approximately equivalent, but it can range from about one-half to three-fold. There are no reliable dermal comparison factors (Table 14).

Table 13: Some Differences
Intravenous vs. Inhalation (IN)

- (IN) dose is gradually acquired (1 or 4 hours);
- During (IN) exposure, detoxification, metabolization, elimination, storage, etc., may occur;
- Lung damage may impair gaseous exchange;
- Toxic effects may increase or decrease due to lung passage;
- Absorption may be incomplete via lungs;
- Pulmonary excretion may not occur.

Table 14: Tentative Conclusions

- Oral $LD_{50} \cong 2 \times IV\ LD_{50}$
- Inhalation $LD_{50} \cong IV\ LD_{50}$ but ranges from 0.5 to 3 x IV LD_{50}
- Dermal $LD_{50} \not\propto IV\ LD_{50}$

All of this information was developed by a large group of people. It included the contributions of chemists and chemical engineers, pathologists, analytical chemists, and toxicologists. Our cholinesterase work is continuing, and we are attempting to correlate biological effects with cholinesterase depression, in relation to the LC or LD_{50}s.

During the coming year, we will use the inhalation system with a selected number of pesticides to investigate possible teratological responses. The exposure regimen will be a fraction of the LC_{50}, an hour per day during the 7th to 15th day of gestation. The animals will be necropsied the day before delivery, with the usual teratological examinations of the fetuses.

We also plan to work with at least one labeled compound to try to follow absorption of the compound across the placenta and into the fetus. For reference teratogens, we will use either chloroform or methylethylketone. Both have shown to produce various terata by inhalation exposure.

STUDIES ON TOXICITY TO MAMMALS OF SMALL PARTICLE AEROSOLS OF NUCLEAR POLYHEDROSIS VIRUS (NPV) PESTICIDES

Dr. James T. Stevens*

NOT AVAILABLE

FOR

PUBLICATION

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*National Environmental Research Center, Research Triangle Park, North Carolina

METABOLISM OF PESTICIDES

Dr. Ronald L. Baron*

Unlike the first two speakers, Jim Stevens and I are surrogates, talking about somebody else's work at the Naval Biomedical Research Laboratory. This is an interagency agreement with the Department of the Army with the work contracted out to the Naval Biomedical Research Laboratory (NBRL) in Oakland, California.

The background to this particular study is as follows: The Army contracted with the Naval Biomedical Research Lab for inhalation toxicology studies on pesticides. As a spin-off from part of their work, Dr. Peter Berteau found some very interesting results with the organophosphorous pesticide naled, or Dibrom, which is a brominated derivative of DDVP. His initial studies indicated that the organophosphate naled has a low toxicity when administered by the oral route. The LD_{50} ranged from 160 to 430 milligrams per kilogram in the adult rat.

Studies with this compound at the NBRL have indicated that by inhalation naled appeared to be quite toxic to the rat, these conclusions being based upon toxicant retention values calculated by the inhalation of a radioactive tracer. The inhalation toxicity to the female rat was greater than 25 times that of the oral toxicity. As you might have seen with Dr. Newell's comparative data, there is roughly a two-fold difference between oral and inhalation toxicity. With naled the 25-fold variation was quite unusual.

Greater inhalation than oral toxicity values are also evident with the mouse, although the differences are less marked with the mouse than with the rat, probably because the rat retained more material in the lung than the mouse. Dr. Berteau and co-workers calculated that the rat retained 6.4 percent of the inhaled toxicant, whereas the mouse retained only 3.5 percent.

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The EPA is currently supporting a program at NBRL to define some of these differences, studying a comparison of the metabolic breakdown of naled in rats, following administration by both the oral and inhalation routes.

Possibilities that can occur to explain the results of the high inhalation toxicity are:

1. The corrosive action of naled on lung tissue; naled is known to be highly corrosive to lung, and lung necrosis has been observed following inhalation exposure. However, the fact that the rats die rapidly, exhibiting marked cholinergic signs of poisoning, indicates that this phenomenon is not the whole story. Corrosive action would also not explain the greater inhalation toxicity to the rat than to the mouse.

2. The possibility of rapid absorption into the blood stream through the lung alveoli, and rapid absorption will occur with all pesticides following inhalation. However, in the case of another organophosphorous compound, chlorpyrifos or Dursban, only a very slight increase in toxicity results following inhalation by the rat; there was none in the mouse. Again, this suggests that naled may have a special property that accounts for its high inhalation toxicity.

3. The difference in the metabolism or metabolism rate to a more toxic intermediate through some mechanism in the lung. In the body, naled can be expected to be metabolized by two routes: hydrolysis, or enzymatic breakdown to dimethyl phosphoric acid, bromodichloro acetaldehyde, and hydrogen bromide. This reaction is rather rapid at alkaline pH.

In the presence of sulphydryl compounds, such as glutathione, naled is rapidly debrominated to produce DDVP, or dichlorvos.

Dichlorvos is also an important insecticide. It is considerably more toxic to mammals than naled. At NBRL the LD_{50} has been determined to be between 56 and 80 milligrams per kilogram, orally, in the rat, whereas, with naled it was calculated to be 160. Therefore, there is a possibility that in the lung, sulphydryl groups may convert naled into dichlorvos, which might explain the enhanced inhalation toxicity.

Preliminary in vitro work with unlabeled naled has demonstrated the presence of dichlorvos using thin-layer chromatography following stirring of a solution of naled with an aqueous solution of cysteine. However, oral intubation of naled to a rat and periodic collection of urine has failed to demonstrate either unchanged naled or dichlorvos in the urine.

These experiments are very preliminary and form the basis for the study that EPA has just funded. NBRL is currently awaiting the availability of 14_C-labeled ethyl naled, the synthesis of which has been contracted, but problems seem to have arisen in the synthesis.

It is easily demonstrated that naled can be synthesized through the production of chloral, the chlorination of ethanol. The reaction of chloral with trimethyl phosphite will produce labeled DDVP, which could be brominated to produce naled. At this particular time, we are still waiting for the preparation. In the future, there will be an examination of the metabolism via both oral and inhalation routes of administration.

As with the previous interagency agreement, this one was started in the latter part of June. Hopefully, by this time next year, we should have results from this program.

DR. CURLEY: These research studies on inhalation toxicology summarize the state-of-the-art in our work with chemical pesticides and also the available facilities of EPA at the Research Triangle Park facility. They point out the need for good inhalation data, as well as the need for well-trained personnel in the area of inhalation toxicology.

We will now open the session for a discussion of these studies. If there are any questions, we'd like to entertain these questions now.

DR. CRANMER: It seems to me that the purpose of inhalation toxicology, or inhalation as a route of exposure, as opposed to a feeding study, is to be more representative of different exposure groups. Now, if we were talking about pesticides and we were talking about residues, the feeding study would be perhaps more appropriate. We then begin to speak of the types of persons that we could be concerned about being exposed, and two groups immediately come to mind -- the person who elects to use a pesticide around the home and the occupationally exposed individual. They would likely be exposed to particulates or aerosols of these compounds. Now, with respect to that, it would seem to me that we should pay an extraordinary amount of attention to explaining not that there's a difference in these toxicities through these different routes, because I think we all agree that there would be differences in this, but to explaining this expression of differences on the basis of two basic groups of phenomena. One would be expression through a different mechanism. Now, it might be a different effect on the lung, or an altered metabolism, or it indeed could be a difference in the absorption kinetics. And now, drawing on Dr. Newell's presentation, I would ask if future work, especially with the anticholinesterase pesticides, would draw on the relative kinetics of inhibition of brain cholinesterase, red cell cholinesterase, and plasma cholinesterase as indexes of these phenomena, and that with parathion, a compound that was discussed, the plasma and red cell are inhibited at differential rates at different levels. I think expressing these things on a relative anticholinesterase potency might give more insight than searching for body burdens or blood levels with these very rapidly metabolized types of compounds. And I would just like to ask if special emphasis is going to be placed on those phenomena in the future?

DR. CURLEY: Yes, that is, I think Dr. Newell or Dr. Baron can answer that. This is the reason we decided against looking at body burdens and we decided to look at the cholinesterase. I think he summarized this in his summary statement. He indicated that he would use cholinesterase determinations as an assessment technique for the toxicity of those compounds via the inhalation route of exposure.

DR. CRANMER: Well, I think it's important to move this up perhaps one more level of sophistication into cholinesterase and its subgroups: the carboxyl esterases, in general, which might be inhibited; the plasma cholinesterase; red blood cell cholinesterase, and then brain cholinesterase. As far as the plasma and red blood cell, it's easy to get 50 microliter samples, which will give you the answer.

DR. CURLEY: Yes.

DR. CRANMER: And one final question, and that is, since we are looking at very specific exposure groups now, are we attempting to relate the components used to generate the aerosols and the types of aerosols that would be generated with the expected exposures that humans actually have? Dimethylsulphoxide, for instance, I don't think that's used — is it? — in any of the commercial formulations, and it has very peculiar absorption kinetics associated with that. And there's the physics which is very interesting being able to relate the ability to absorb certain size particles with different velocities in the lung. I don't think that's well enough worked out. An interspecies comparison would be a very important contribution. What are the velocities of the aerosol that's available to be trapped? I'd just like to throw some of those points open for discussion.

DR. CURLEY: I think these questions that Dr. Cranmer asked have been raised by us, by Dr. Newell, and by the people in inhalation studies at the Naval Biomedical Research Laboratory. Dr. Cranmer raised the question concerning the effect of physical chemical properties, that is, particle sizes and velocity of these particles. We've also raised the same questions with Dr. Newell, including the use of various vehicles and the effects of these vehicles on the toxicity of the compound, and whether there is a synergistic or potentiation effect by use of, say, DMSO as a vehicle to generate the aerosol. Hopefully, these questions will be answered.

We have given consideration to those studies. Dr. Stevens, I think, has plans although he did not mention it in his presentation, to institute studies at our laboratory to assess the physicochemical properties of the particulates or of varying the particle sizes, etc. These pesticides were generated as aerosols. He used the term toxicokinetics, or pharmacokinetics, which includes rates of absorption, lipid solubilities, and the effects of particle size and that sort of parameter. I think a great deal of this work has been done with anesthetics through intratracheal injections of compounds directly into the respiratory tree. But this kind of work has not been done via the nasopharyngeal region and, therefore, we must assess that aspect of pesticide inhalation, including the vehicles in which these compounds are generated as aerosols. There is a possibility that any of these parameters might contribute to the toxicity of a compound.

DR. NEWELL: Dr. Cranmer commented that it is desirable to study compounds in the neat form. Dr. Cranmer, we talked about this earlier. It has been the opinion of the EPA staff and ourselves that you should obtain the initial information on the technical product. You could go back, and if it appears feasible, look at the vehicle or the carrier itself and also the neat material to see if there is potentiation or inhibition. In this manner, you could obtain relative toxicological information. Whether or not we go in that direction is problematical. Perhaps the manufacturers of the compounds will do this themselves.

DR. ENGLER: I have a question with regard to the presentation on the viral pesticides. Why has the Heliothis virus been chosen to be tested again? There are some inhalation toxicity studies available on that virus. Maybe they were not conducted with the same degree of sophistication as these current studies but they are adequate. I would like to point out or urge therefore that other viruses which may have the potential of becoming useful control agents are just as important to be studied as the Heliothis virus, for which we have considerable data on toxicity or infectivity available.

I also would like to point out that although we are talking mostly about chemicals at this conference, we should strike the word toxicity when we consider insect viruses. We are not concerned about their toxicity but rather their infectivity and other effects attributable to microorganisms and, of course, serological responses. Therefore, right from the start, when we include the viruses in the substitute chemicals, we should be aware that they are not to be treated as chemicals.

DR. CURLEY: Dr. Engler of OPP asked the question, "Why was the nuclear polyhedrosis virus selected?" It was selected as an extension of an in-house effort. Presently, to our knowledge and perhaps to your knowledge, there may be other manufacturers in the country other than Nutrilite in California which is producing viruses other than the nuclear polyhedrosis viruses. The product Nutrilite produces is a virus. As Dr. Stevens pointed out, insect parts, fungi, and just about a little bit of everything has been found, including a lot of ash, indicated by analysis by two groups of researchers -- one at Scripps and the other at the NBRL in California. We would appreciate any studies or any data that you could make available to us on viruses other than those, Dr. Engler.

DR. KAMIENSKI: I'm still trying to evaluate what the significance of these inhalation studies is as they would apply to making a hazard evaluation as to what the workers in the field are exposed to. I don't know if there are follow-up studies provided for this area or not. My question is, are there any inhalation studies that are being considered using the particle size range that is normally encountered or being generated by agricultural equipment? In other words, you've identified the toxicity of some of these materials, and this is with particle size ranges that are 95 percent respirable, as opposed to inhalation studies using the equipment that is normally used in agricultural sprays which generate a much larger particle size, where the respirable particles are probably less than 1 percent.

The other question is, can you explain to me the logic of running teratogen studies via the subacute inhalation route?

DR. CURLEY: I'll attempt to answer the last question. I'll ask Dr. Dale to answer the first.

DR. DALE: Number one, this study, as I envisioned it, wasn't attempting to study the hazard of pesticides as exposed to field workers. It was to generate aerosol to get the most toxic form, so we could compare routes of exposures -- inhalation versus dermal versus oral versus intravenous. And, in this case, aerosol was used because you get the most respirable particle and the most toxicity on this route. No, this cannot be extrapolated to field hazard from spraying equipment. Although I wonder if it could not be extrapolated to vapors arising on reentry problems, because in this case the particles are certainly in the small range. But to answer your first question, it was an attempt to determine hazard from spray equipment or from dust.

DR. CRANMER: I was speaking of this in my usual kind of nebulous style a minute ago, but what I was trying to say was, if one takes a certain particle size which is consistent with what perhaps a worker would be exposed to, then one tries to model after that a very small rodent, then the trajectory physics associated with the impacting of that particle, the absorption of that aerosol by the lungs or other associated paraphernalia of the inhalation route is quite different. So what we really have here is a modeling problem, where we really need to base it on relative capability to absorb or be absorbed by, rather than an exact replica of particle size. I think we'd fall into some gaping holes if we tried to do it that way. The physical model though, to my knowledge, is not worked out very well. Some particular attention will be necessary in that area if we're to be able to make that bridge.

DR. CURLEY: Yes, that's correct. Now, to your second question, there are several inconclusive reports in the literature on several of the insecticides, principally the organophosphate ester type compound that suggests there may be possible teratogenic effects. These are reports -- they're basically medical reports in medical journals -- that indicate housewives, or whoever, or migrant female field workers were exposed to these compounds and children with certain types of malformities were born to these mothers. However, these reports are inconclusive and, therefore, they suggested a need to evaluate the possibility of producing terrata in animals via the inhalation route, because the apparent route of exposure to these females was the inhalation route. I think that this was the question that you raised. And therefore Dr. Newell will extend his studies to evaluate that possibility.

TOXICOLOGICAL EVALUATIONS

Dr. Ralph I. Freudenthal*

A number of registered pesticides are presently being reviewed by EPA's Office of Pesticide Programs. One phase of this review is to evaluate the existing toxicity data for each pesticide and identify and define areas of missing data. On June 17, 1974, Battelle's Columbus Laboratories was awarded a toxicology contract to help fill these data gaps by initiating research programs of a high-priority nature.

In order to fulfill the toxicity testing needs of EPA's Office of Pesticide Programs, we are providing the following capabilities:

1. Acute and subacute feeding studies;
2. Oral and dermal exposure studies in small mammals;
3. Pharmacokinetic studies in small mammals and in subhuman primates — this includes measuring the rate of absorption, whole-body distribution, metabolism, and rate of excretion;
4. Studies to determine the effect of the pesticide on enzyme activity;
5. Multigeneration reproduction studies; and
6. Neurophysiological and behavioral studies.

The general scope of our work is divided into two major areas:

1. Response to inquiries for data from ongoing review processes; and
2. Response to inquiries for data on compounds identified as impurities in technical pesticide formulations.

Although the contract was awarded in June 1974, no research tasks were assigned to Battelle until February 1975. Because of this time lag between contract award and task assignment, I cannot present to you the results from any completed study, but I will present a brief overview of the research tasks presently assigned to our laboratory.

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*Battelle Memorial Institute, Columbus Laboratories, Columbus, Ohio

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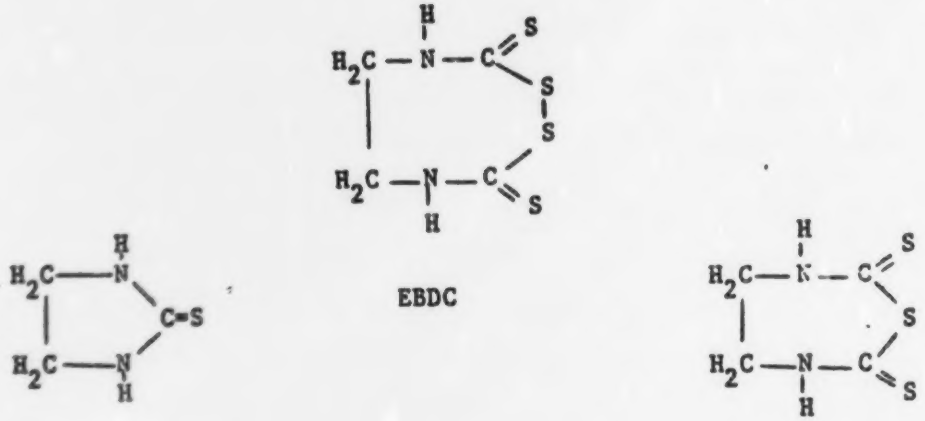
Task 1. Ninety-Day Toxicity Study of Ethylenebisisothiocyanate Sulfide (EBIS)

It is now well documented that the ethylenebidithiocarbamate (EBDC) fungicides decompose to form ethylene thiourea (ETU). ETU exhibits goiterogenic as well as dose-related carcinogenic properties. The EBDC fungicides also degrade to EBIS, whose toxicity and carcinogenicity are presently unknown. (See Figure 1.)

Figure 1



ETU

EBDC

EBIS

Our assigned task is to perform a 90-day EBIS toxicity study in rats, in which the test chemical is incorporated into the feed. Unfortunately, there is no commercial source of EBIS. Therefore, a custom synthesis of EBIS is underway at Trans World Chemicals, with an estimated delivery date of August 1. The study will be initiated upon receipt of the chemical and its incorporation into the feed.

Since ETU is goiterogenic, ^{125}I uptake studies and T_3 and T_4 tests will be performed on the rats. After reviewing the T_3 and T_4 kits from Ames, Squibb, Oxford, Abbott, Pharmacia, and Nuclear Medical, we chose the Nuclear Medical kits, our choice being based on their sensitivity, assay time, and components. In a pilot study to test the Nuclear Medical kit, we used serum from 10 control rats and from 5 rats which were treated with methimazole, a thiocarbamate type antithyroid compound. The results are shown in Table 1.

Table 1: Rat Serum Assays

Group	$T_3 \pm$ S.D.	$T_4 \pm$ S.D.
Control	63.12 ± 1.16	4.66 ± 1.18
Methimazole	63.13 ± 2.07	1.95 ± 0.84

The data clearly show that the TBG levels were not affected by the drug, whereas the concentration of thyroid hormone was substantially affected. We are now convinced that the Nuclear Medical kit is suitable for measuring T_3 and T_4 levels in rat serum.

Task 2. Determine the Degradation of Selected EBDC Fungicides under Various Cooking Conditions and on Certain Fresh Vegetables by Measuring Quantitatively EBDC, ETU, and EBIS

Initially, this study was to be performed in our laboratories. For a number of reasons, a subcontract was awarded to Technological Resources, Inc., a subsidiary of the Campbell Soup Company, who will perform the following studies:

1. Develop a quantitative assay for measuring EBIS;
2. Determine residue levels of EBDC, ETU, and EBIS on three crops following the application of Dithane M-45 and Dithane M-22;
3. Determine the effects of normal washing and preparation on residual EBDC, ETU, and EBIS;
4. Determine the effects of food processing procedures (canning and freezing) on residual EBDC, ETU, and EBIS; and
5. Determine the rate of formation of ETU and EBIS under different cooking conditions (pH, ionic strength, etc.)

Technological Resources, Inc., is presently working on the EBIS assay. In addition, they have harvested and processed the spinach grown in New Jersey. Analysis of the Dithane M-45-treated spinach samples for EBDC and ETU is also in progress.

Task 3. Pharmacokinetics of Pentachloroaniline in the Rhesus Monkey

Pentachloroaniline (PCA) is a metabolite of the soil fungicide, pentachloronitrobenzene (PCNB). It has been identified in the body fat, liver, and feces of a number of animal species to which PCNB was administered. In addition, PCA has been isolated from cow's milk and from grass grown in PCNB-treated soil.

We have been asked to determine the rate and extent of absorption of PCA by the oral route, its metabolism, and its rate of excretion. To carry out this study, we asked California Bionuclear Corporation to custom synthesize 1 mCi ^{14}C-PCA. We have just received the labeled PCA and will begin this task immediately.

A single dose of ^{14}C-PCA will be orally administered to five male rhesus monkeys and then blood, urine, and feces samples will be collected at specified time points. These biological samples will be analyzed for PCA and its metabolites.

Task 4. Pharmacokinetics of Pentachlorobenzene in the Rat and Rhesus Monkey

Pentachlorobenzene (PCB) is an impurity found in essentially all commercial preparations of pentachloronitrobenzene fungicides. A recent study of certain halogenated benzenes showed that PCB accumulated in the fetus to a greater extent than did PCNB and the other test compounds.

We have been asked to determine the pharmacokinetics of PCB in the rat after oral and dermal administration, and also in the rhesus monkey after oral administration. California Bionuclear Corporation is presently custom synthesizing 1 mCi ^{14}C-PCB for use in these experiments.

In summary, we are just beginning the experimental work recently assigned to us as part of our toxicology contract.

TOXICOLOGICAL RESEARCH: ACUTE LD_{50} STUDIES*

Dr. Larry L. Hall**

Pursuant to the Federal Insecticide, Fungicide, and Rodenticide Act, as amended, and Public Law 93-135, EPA is charged with the responsibility for the evaluation and testing of alternate (substitute) chemicals such that the substitute is not more hazardous to man and his environment than the "problem" pesticide which it is to replace. In order to meet this mandate, our laboratory has initiated a program of research to provide a data base for the gaps that exist in our knowledge of the biological activity of these alternate chemicals. Included in this repertory is our ability to perform a variety of acute, subacute, and chronic studies, including metabolic, kinetic, and reproductive experiments.

Because of the "man or mouse" difficulty, we use a longitudinal approach such that all experiments are performed on the Sherman strain rat so that comparative analysis on pharmacodynamic and pharmacokinetic studies can be performed on different compounds without the introduction of additional variables such as species and strain in order to provide more meaningful input into the review process.

This report presents the acute toxicity study results obtained preparatory to more extensive studies of these compounds in the Alternate Chemicals List.

Methods

Non-fasted adult (90-120 da.) and weanling (30-34 da.) Sherman rats (COBS) obtained from the Center for Disease Control colony were used for these studies.

The solutions, formulated just prior to dosing, were of concentrations such that a (relatively) constant volume (0.005 ml/g) could be administered by gavage. In some instances (pentachloronitrobenzene) limited solubility and low order of toxicity required the use of up to 0.02 ml/g. All dosing was performed between 10 and 11 a.m.

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*Co-authors are R. Linder, W. Felsenstein, J. Goldstein, and A. Curley

**Chief, Acute and Chronic Studies Section, Environmental Toxicology Division, Research Triangle Park, North Carolina

For the dermal toxicity study, pentachloronitrobenzene (PCNB) was dissolved in xylene and applied to the skin at a volume of 0.01 ml/kg after clipping the hair. No attempt was made to prevent oral ingestion.

All compounds used were technical formulations obtained from contract except for the PCNB used in the suspension studies which was obtained commercially (Table 1.)

Table 1: List of Common and Chemical Names of Compounds Tested

Common Name	Chemical Name	Purity	Use
Captan	N-[(Trichloromethyl)thio]-4-cyclohexene-1,2-dicarboximide	Technical	Fungicide
Folpet	N-(Trichloromethylthio)phthalmide	Technical	Fungicide
Guthion	[azinphos-methyl] *O*,*O*-Dimethyl S-[(4-oxo-1,2,3-benzotriazin-3(4*H*)-yl)methyl] phosphorodithioate	Technical	Insecticide
Fenthion	*O*,*O*-Dimethyl *O*-[3-methyl-4-(methylthio)phenyl] phosphorothioate	Technical	Insecticide
Dicamba	3,6-Dichloro-*o*-anisic acid	Technical	Herbicide
PCNB[1]	Pentachloronitrobenzene 95%	Technical	Fungicide
PCNB[2]	Pentachloronitrobenzene 99+%	Technical	Fungicide
MSMA	Monosodium methanearsonate	Formulation 58.4% active ingredient	Herbicide
DSMA	Disodium methanearsonate	Formulation 80.1% active ingredient	Herbicide
Cacodylic acid	Dimethylarsinic acid	Formulation 65.6% active ingredient	Herbicide

Following dosing, the rats were observed frequently for signs of intoxication. The animals were weighed weekly until termination of the study. Gross autopsies were performed on dead or moribund animals and at the end of each study.

The LD_{50} values were calculated by the method of Finney (1) or Litchfield and Wilcoxon (2) when convergence did not occur in the regressional analysis.

A subacute feeding study was undertaken with pentachloronitrobenzene. Formulation for the feeding study (PCNB) was accomplished by preparing a concentrate of the chemical in powdered ration and serial dilution with additional chow to the levels of concentrations required. The formulation was prepared at tri-weekly intervals.

Six groups of four female weanling Sherman rats were fed technical-grade (98 percent) PCNB for 101 days. The dietary levels were 0, 50, 100, 200, 400, and 800 ppm. Growth rate, hematological, tissue, and biochemical examinations were made.

Liver specimens and urine were taken for biochemical studies. Urine was assayed for aminolevulinic acid, porphobilinogen, coproporphyrin, and uroporphyrin. Liver was assayed for total porphyrin, microsomal protein and P-450, aryl hydrocarbon hydroxylase, and N-demethylase.

Selected tissues were taken for histopathological study.

Results

MSMA or DMSA

Signs of toxicity in rats given MSMA or DSMA included diarrhea, depression, and dyspnea with most deaths occurring in 2-3 days. The predominant finding at autopsy was mild to severe irritation of the gastric mucosa. Cecal contents were mucoid and yellowish. In addition, most rats dying from MSMA had hemorrhagic adrenals and small spleens.

Cacodylic Acid

Signs of toxicity in rats dosed with cacodylic acid were diarrhea, depression, and dyspnea with most deaths occurring 3-5 days after dosing. Gross inspection of dead rats revealed moderate to severe irritation of the gastric mucosa, hemorrhagic adrenals, and atrophic spleens. Tissues of animals given a nonlethal dose (1500 mg/kg) were grossly normal after 14 days.

Table 2: Acute Toxicity of Substitute Chemicals in the Sherman Rat

Compounds	Route[1]	Sex	Age[2]	LD_{50} (mg/kg) 95% Confidence Limits	Slope of Probit Regression Line	Highest Nonlethal Dose Tested[3] (mg/kg)	LD_{50} Literature-TSL[4] (mg/kg) Rat
MSMA [58.4%]	0	F	W	1743 [1589-1895]	10.52	1200	700
DSMA [80.1%]	0	F	W	1695 [1507-1876]	10.10	1200 (1/10)	1800
Cacodylic acid [65.6%]	0	M	A	1802 [1640-1964]	9.16	1000	1350
Guthion	0	F	W	18.3 [16.8-20.1]	13.19	12.5	-
Fenthion	0	M	W	284 [244 -338]	5.87	-	215
Dicamba	0	M	W	3288 [2979-3643]	9.45	1650	1040
Folpet	0	M	W	5000	-	5000 (1/10)	10000
Folpet	0	F	W	5000	-	5000	-
Captan	0	M	W	5000	-	5000 (1/10)	480
Captan	0	F	W	5000	-	5000	
PCNB (99%)	0	F	W	1359 [1254-1461]	16.88	900	1650
PCNB (99%) Suspension	0	F	A	1989 [1890-2101]	20.36	1500	
PCNB [95%] Suspension	0	M	A	2550 [2257-2882]	-	2000	
PCNB [95%] Suspension	0	M	W	2812 [2691-2931]	31.25	2250	
PCNB [95%] Suspension	D	M	A	5000	-	5000	
PCNB (95%) Suspension	D	F	A	5000	-	5000	

[1] Oral [O]; Dermal [D].

[2] Weanling [W]; adult [A].

[3] ( ) Mortality at lowest dose.

[4] Toxic Substances List (1974).

Guthion

Weanling female rats showed typical cholinergic signs, i.e., salivation, muscle fasciculations, lacrimation, and dyspnea with onset a few minutes after dosing. Most deaths occurred within 2-3 hours. Recovery of poisoned animals usually occurred within 20 hours.

Fenthion

Weanling male rats displayed typical cholinergic signs as described for guthion. The onset of signs was observed less than 2 hours after dosing with most deaths occurring between 24 and 48 hours. Survivors recovered in 48-72 hours.

Dicamba

Weanling males displayed dyspnea and generalized myotonic type responses when disturbed. These signs occurred within a few minutes after dosing and persisted for 24-48 hours. Most deaths occurred within 24 hours.

Captan and Folpet

The sign of poisoning in rats given folpet or captan was mild to moderate diarrhea which in most animals persisted 2-4 days after dosing. A few rats exhibited abdominal distension.

Pentachloronitrobenzene (99 Percent Pure)

Signs of toxicity in rats dosed with PCNB included diarrhea, depression, dyspnea, weakness, and tremors when agitated. Most mortalities occurred 1-3 days following treatment. Gross inspection of dead rats revealed hemorrhage into the lumen of the gastrointestinal tract. Tissues of rats surviving on LD_{20} doses (1200 mg/kg) were grossly normal.

Pentachloronitrobenzene (Technical)

Oral and dermal LD_{50} determinations for 95 percent technical PCNB in Sherman rats were completed. The material, because of an unexplained solubility problem, was given as a suspension in peanut oil for oral dosing. Signs of toxicity in orally dosed animals were labored breathing, depression, and diarrhea, followed by excitability in rats that recovered. Most deaths occurred 2-3 days after dosing. No definite signs of toxicity were seen in dermally exposed animals.

PCNB - Pilot Subacute Study

No signs of toxicity were observed in the test animals. Growth rates in the test groups were comparable to those of controls. No change in organ weights was observed in the test groups. Hematological profiles of the test groups did not differ from those of the controls.

The only definite changes observed grossly at autopsy were moderate lesions of both kidneys in one rat at the 200 ppm dietary level. This finding is probably not dose-related. Histological examination is not complete at this time.

Liver samples were taken from all rats at the termination of the experiment for biochemical studies. No significant changes were noted in the total liver porphyrin or microsomal protein concentration. Urinary aminolevulinic acid, coproporphyrin, or uroporphyrin were not changed. The data were suggestive of a slight increase in porphobilinogen at the higher dietary levels. Liver P-450 and N-demethylase appeared slightly elevated at the 800 ppm level. Aryl hydrocarbon hydroxylase appeared elevated at 100 ppm and above.

Discussion

Reasonable agreement exists between our LD_{50} values and those recorded in the literature in spite of the possibility of quite different degrees of purity. The exceptions were MSMA and captan. Captan, folpet, and dicamba exhibited relatively low orders of acute toxicity.

Relative toxicity probit analysis of the organic arsenicals revealed no significant differences between the formulations in spite of a rather wide range of active ingredients (58.4 - 80.1 percent). The similiar slope values suggest similiar mechanisms of acute toxicity.

Guthion was found to be 15.5 times more potent than fenthion and showed a much steeper dose-response relationship. The reason for this phenomenon is not understood.

The battery of acute PCNB studies suggests a sex difference with females being more sensitive than males. However, no significant effect of age was found in male rats. A difference between suspensions and solutions of the agent is also suggested.

No grossly significant toxicological effects were noted at any of the dose levels in the PCNB feeding study. The importance of the biochemical changes noted is unknown at this time. A chronic study is needed to evaluate the significance of these early changes. In addition, the possibility of the contaminant being the causative agent needs to be ruled out.

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2-AAF AS A MODEL COMPOUND

Dr. Thomas J. Haley*

Following the selection of the chemical 2-acetylaminofluorene, the mouse strains (BALB/c and C57BL/6) and a thorough review of the literature on the toxicity of 2-AAF, it became apparent that an acute oral LD_{50} determination and a subacute 90-day feeding study were required before the beginning of the ED_{01} experiment was attempted.

2-AAF as a suspension in aqueous 10 percent polysorbate-80 was administered orally to the various mouse strains shown in Figure 1. Symptoms of acute toxicity were lethargy, sedation, loss of righting reflex, circling, orange-red urine, bladder concretions, and paralysis with all four limbs rigidly extended. In can be seen that, generally, the females were more susceptible to the toxic effects of 2-AAF. Moreover, both groups of C57BL/6 female mice showed significant differences from the males (Table 1). Experiment No. 1 CD-1 males showed significantly lesser toxicity than those in the other experiments (Table 2). Comparisons of responses between No. 2 CD-1 vs. BALB/cj; C57BL/6Cr vs. C57BL/6j or BALB/cj; and C57BL/6j vs. BALB/cj showed no significant differences, while all other comparisons between groups were significantly different. Comparison between all-female groups and No. 1 CD-1 showed significant differences in response (Table 3). No significant difference in response was found when comparison was made between No. 2 CD-1 vs. C57BL/6Cr or BALB/cj female mice. Bladder concretions were present in 7.5 percent of the animals and appeared at 48 hours. Of greatest importance was the difference in responses between animals of the same strain from different sources.

In the 90-day subacute feeding study, 1200 mice, each of the C57BL/cj and BALB/cStCrlBR strain, equally divided between the sexes, were used. 2-AAF concentrations in the diet were 500, 250, 100, 50, and 10 ppm. An unbalanced statistical design was used with groups of 80, 80, 160, 240, and 240 for the above concentrations. A control group contained 240 animals. Histopathological examination was performed on the lungs, heart, aorta, thymus, skeletal muscle, kidney,

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Table 1: Oral LD_{50}/7 Days of 2-FAA in Several Mouse Strains

Expt no.	Strain	No. mice	Sex	LD_{50} and range[a] (g/kg)	Slope and range[a]
1	CD-1	100	M	2.02 (1.89-2.16)	1.19 (1.07-1.32)
	CD-1	100	F	1.87 (1.75-2.00)	1.20 (1.06-1.36)
2	CD-1	100	M	1.08 (1.02-1.14)	1.17 (1.06-1.29)
	CD-1	100	F	1.08 (1.02-1.14)	1.17 (1.06-1.29)
3	C57BL/6Cr	100	M	1.22 (1.16-1.28)	1.11 (0.98-1.25)
	C57BL/6Cr	100	F	1.02 (0.96-1.08)	1.13 (1.0 -1.28)
4	C57BL/6j	100	M	1.23 (1.17-1.29)	1.11 (0.98-1.25)
	C57BL/6j	100	F	0.81 (0.76-0.86)	1.18 (1.07-1.3)
5	BALB/cj	100	M	1.20 (0.98-1.46)	1.17 (0.84-1.64)
	BALB/cj	100	F	1.17 (1.08-1.27)	1.26 (1.09-1.46)

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[a] All values at p=0.05.

Table 3: Comparison of LD_{50}/7 Days Between Females of Different Strains[a]

Strain	No. 1 CD-1	No. 2 CD-1	C57BL/6Cr	C57BL/6j	BALB/cj
No. 1 CD-1	—	S	S	S	S
No. 2 CD-1	S	—	NS	S	NS
C57BL/6Cr	S	NS	—	S	S
C57BL/6j	S	S	S	—	S
BALB/cj	S	NS	S	S	—

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[a] S=significantly different at p=0.05; NS=not significantly different.

Table 2: Comparison of LD_{50}/7 Days Between Males of Different Strains[a]

Strains	No. 1 CD-1	No. 2 CD-1	C57BL/6Cr	C57BL/6j	BALB/cj
No. 1 CD-1	—	S	S	S	S
No. 2 CD-1	S	—	S	S	NS
C57BL/6Cr	S	S	—	NS	NS
C57BL/6j	S	S	NS	—	NS
BALB/cj	S	NS	NS	NS	—

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[a] S=significantly different at p=0.05; NS=not significantly different.

adrenal, liver, spleen, gallbladder, pancreas, cerebrum, cerebellum, spinal cord, stomach, colon, ileum, duodenum, lymph node, salivary glands, lacrimal gland, eye, hardeian gland, thyroid, trachea, esophagus, skin, tongue, sternum, testes, epididymus, preputial gland, seminal vesicle, coagulating gland, ovary, uterus, mammary gland, urinary bladder, prostate, pituitary, and any unidentified mass.

All data were collected and stored in a Modular Computer Systems III/5 minicomputer. No observable signs of toxicity were produced in either strain or sex of mice. At 500 ppm, a slight nonsignificant weight retardation occurred in both strains, with a greater effect on the C57BL/6 males and the BALB/c females. Figure 1 shows the dose-related bladder hyperplasia in BALB/c mice; it was the same for the C57BL/6 strain. The 100 to 500 ppm gave a 100 percent response. The BALB/c males had a bladder metaplasia double that of the females which was also dose-related. One BALB/c male mouse had a bladder carcinoma at 500 ppm and another at 250 ppm. Adrenal spindle-cell hyperplasia was seen in both strains. Males of both strains had urinary bladder concretions.

When one considers the 90-day subacute study, it is apparent that the dosage for urinary bladder hyperplasia was too high; therefore, a serial sacrifice experiment was designed to study this response. Also another question arose of whether 2-AAF exposure had to be continuous or if a 3-month exposure would lead to carcinoma 9 months later. Three separate experiments employing 1680 BALB/c mice, each with an equal distribution of the sexes, were used. The doses fed were 8, 24, 45, 59, and 86 ppm, with the mice distributed as follows: 480, 480, 240, 160, and 80 animals per dosage level. The same tissues examined in the other experiment were examined in these experiments. The sacrifice periods were first experiment - 1, 3, 6, 9, and 13 weeks; second and third experiments - 3, 5, 7, 9, and 12 months. In the second experiment, exposure to 2-AAF ceased at 3 months and in the third it was continued for the total 12-month period.

Figure 2 shows the hyperplastic response obtained in the males. Whereas hyperplasia developed in the females only at the two higher doses and at the 9- and 12-month sacrifice periods, hyperplasia in the male made its first appearance at

Figure 1

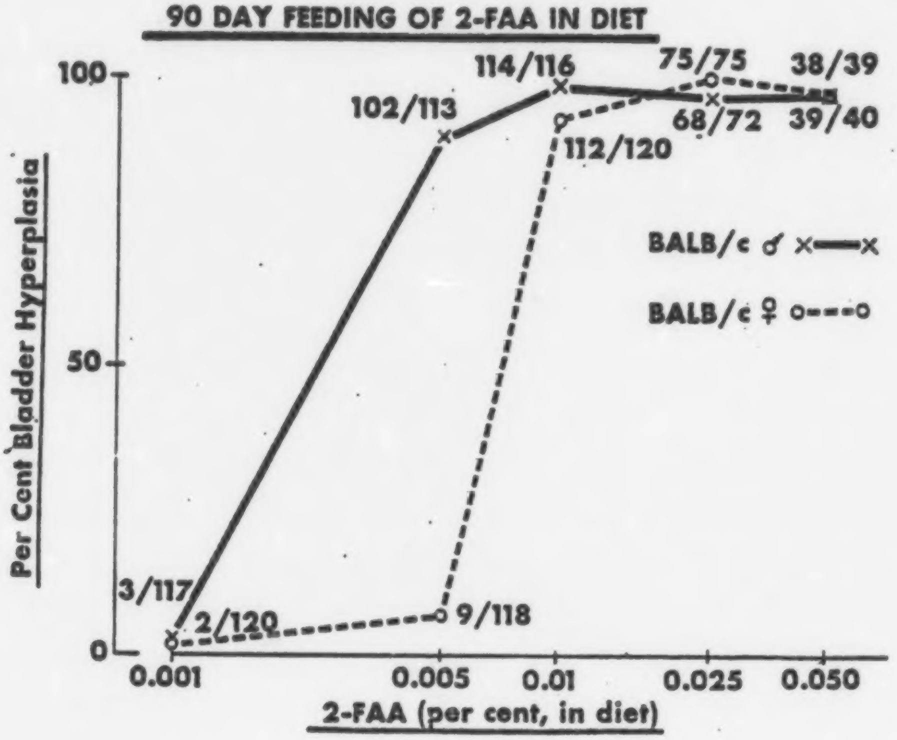


Figure 2

COMPARISON OF TOXICITY, MALES vs. FEMALES SAME STRAIN

M / F	#1 CD-1	#2 CD-1	C57BL/6Cr	C57BL/6j	BALB/cj
#1 CD-1	NS	—	—	—	—
#2 CD-1	—	NS	—	—	—
C57BL/6Cr	—	—	S	—	—
C57BL/6j	—	—	—	S	—
BALB/cj	—	—	—	—	NS

M= males; F= females

S= Significantly different at P= 0.05

NS= Not significantly different

Figure 3

Incidence of Urinary Bladder Hyperplasia in BALB/c Strain Mice Fed N-2-Fluorenylacetamide

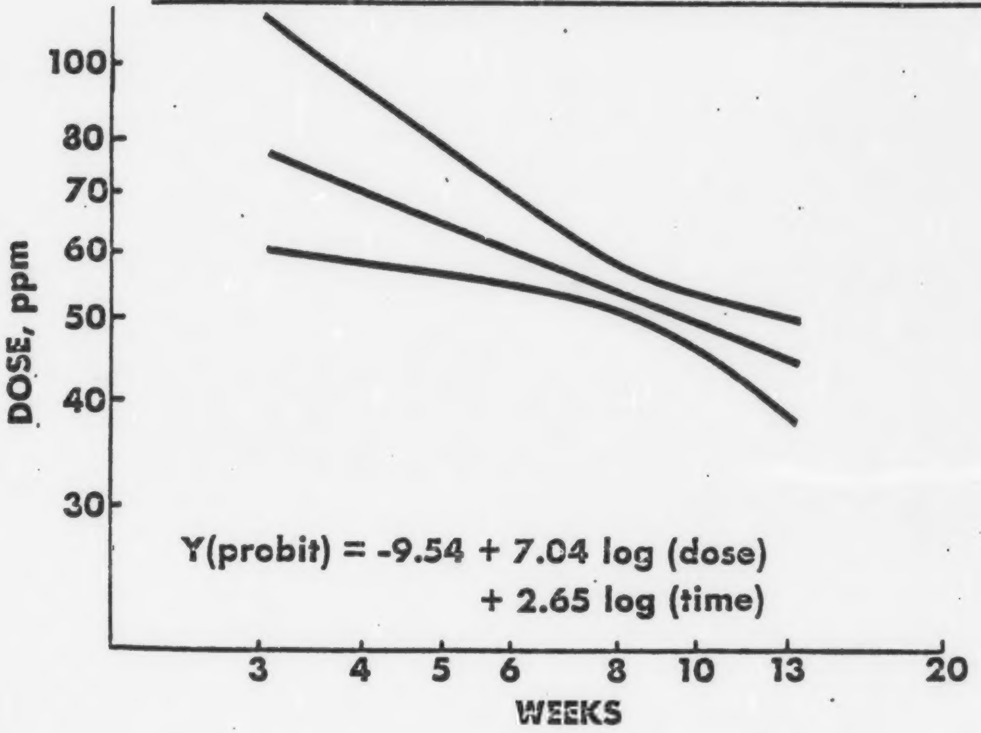
Sacrifice Interval, weeks	Sex	Dose of N-2-Fluorenylacetamide, ppm					
		0	8	24	45	59	86
1	♂	0/24[a]	0/48	0/48	0/24	0/16	0/8
	♀	0/24	0/48	0/48	0/24	0/16	0/8
3	♂	0/23	0/47	0/48	1/24	2/16	6/8
	♀	0/24	0/48	0/48	0/24	0/16	0/8
6	♂	0/24	0/47	0/48	2/24	11/16	8/8
	♀	0/24	1/47[b]	0/47	0/24	0/16	0/8
9	♂	0/23	0/47	2/48	5/22	11/15	8/8
	♀	0/24	0/47	0/46	0/24	0/16	2/8
13	♂	0/24	0/46	3/44	7/23	13/15	8/8
	♀	0/24	0/47	0/47	0/24	2/16	3/8

[a] Number responding out of total number in group.

[b] Animal had cystitis.

Figure 4

A plot of 5.00 = –9.54 + 7.04 log (dose) + 2.65 log (time) with appropriate confidence limits on dose.



$$Y = a + bX + cZ \qquad \text{(Eq. 1)}$$

$$Var(Y) = 1/Snw + x^2/Snwx^2 + 2xz/Snwxz + z^2/Snwz^2 \qquad \text{(Eq. 2)}$$

$$x = X - \overline{X} \qquad \text{(Eq. 3)}$$

$$z = Z - \overline{Z} \qquad \text{(Eq. 4)}$$

By replacing $Y = \overline{Y} + bx + cz$

$$X = \overline{X} + \frac{-B \pm \text{root}\,(B^2 - 4AC)}{2A}$$

where

$K = \overline{Y} + cz - Y_O$

$A = b^2 - t^2/Snwx^2$

$B = 2(K - t^2 z/Snwxz)$

$C = (K^2 - t^2/Snw - t^2 z^2/Snwz^2)$

Y = Probit of response

$X = \text{Log}_{10}$ (dose)

$Z = \text{Log}_{10}$ (time)

Figure 5

Hyperplasia

		Months				
Males		3	5	7	9	12
	86	6/8	8/8	7/7	8/8	8/8
	59	14/16	13/16	13/14	13/15	12/13
PPM	45	20/24	21/23	19/24	21/24	18/22
	24	11/48	7/45	16/48	15/42	17/44
	8	—	1/46	1/48	1/44	6/41
Females						
	86	5/8	8/8	8/8	8/8	8/8
	59	2/16	6/15	7/12	10/15	6/16
PPM	45	—	1/23	4/28	0/25	—
	24	—	—	—	1/46	—
	8	—	—	—	—	—

Figure 6

CARCINOMA

		Months				
Males		3	5	7	9	12
	86	—	—	—	3/8	4/8
	59	—	—	—	—	3/13
PPM	45	—	—	—	—	1/22
	24	—	—	—	—	—
	8	—	—	—	—	—

3 weeks and increased thereafter except at the lowest dose. No hyperplasia was observed in the control groups. A statistical analysis of this male data (Figure 3), using a maximum likelihood solution, shows that it is possible to take a fixed response and using a linear equation to solve for dose for a given time or a time for a given dose and confidence limits placed on either (Figure 4). The only other pathological change observed was a spontaneous pericardial fibrosis and mineralization which occurred in all groups, even in the controls. It was seen in 26.4 percent of the males and 13.8 percent of the females.

In the second experiment, where feeding 2-AAF ceased at 3 months, hyperplasia cleared up at 5 months and neither it nor carcinoma was observed throughout the remaining months of the study. In the third experiment, where 2-AAF was fed for 12 months, urinary bladder hyperplasia was observed in both males and females, with this response appearing earlier in the former (Figure 5). Bladder carcinoma appeared in the three highest doses of the males but not in the females (Figure 6). Both the bladder hyperplasia and neoplasia are dose- and sex-dependent and it appears that hyperplasia is related to total dose consumed and not to dose rate.

In summary, 2-AAF is a compound with a very low acute oral toxicity. When administered subacutely, it produces a dose-related urinary bladder hyperplasia which is sex-related; males are more susceptible than females. Bladder carcinoma also was sex-related, occurring only in males. Bladder concretions also occurred only in males. Adrenal spindle-cell hyperplasia was observed in both BALB/c and C57BL/6 strain mice. Spontaneous pericardial fibrosis and mineralization were observed in male and female BALB/c mice.

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A STUDY OF AGE SENSITIVITY TO THE CHEMICAL CARCINOGEN 2-ACETYLAMINOFLUORENE*

Dr. David L. Greenman**

The appearance of tumors during chronic administration of a chemical carcinogen frequently occurs only after an extended period of exposure to the carcinogen. In many instances it is unclear whether the length of the "latent period" is inherent to the carcinogenic process or is related to factors outside of carcinogenesis per se. For example, numerous developmental and aging changes are known to occur from the time of birth until death of any given animal. Such changes range all the way from alterations in the immunological capacity of the animal to changes in the concentration and/or inducibility of certain liver enzymes. Conceivably almost any of the physiological or biological changes that occur with aging could bring about a change in an animal's response to a given carcinogen. In general, studies of the effect of age on the sensitivity of animals to chemical carcinogens have compared neonatal animals with older animals but have neglected to compare animals treated at intermediate times between weaning and old age.

The major objectives of this study were 1) to detect differences in susceptibility to induction of urinary bladder tumors by 2-acetylaminofluorene (2-AAF) at three different post-weanling periods in the life of BALB/c female mice, and 2) to determine whether progression of these tumors following withdrawal of the carcinogen is an age-dependent phenomenon.

The BALB/c female mice used in the experiment were specific-pathogen-free animals, cesarean-derived, and produced at the National Center for Toxicological Research. They were approximately 4 weeks old at the start of the experiment and were housed four mice per cage. The animal room was maintained as a clean conventional room

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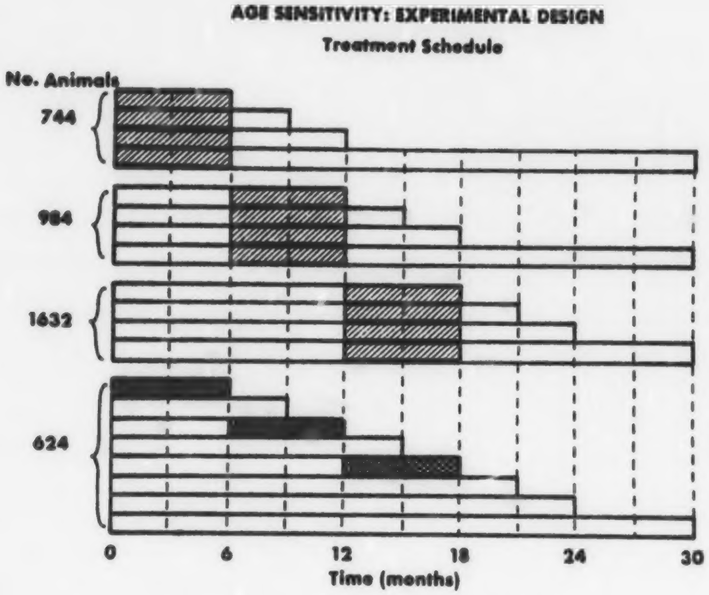
*Co-authors are A. Booth and C. Frith

**National Center for Toxicological Research, Jefferson, Arkansas with entry restricted to those associated with the experiment. A face mask, gloves, cap, clean coveralls, and clean shoes were required apparel for those who entered. Animals, soiled bedding samples, and drinking water samples were routinely removed from the room for diagnostic analysis to detect the entry of pathogens into the colony.

Pelleted autoclavable diet was used during periods when mice were on control feed. Autoclavable meal was used for some of the control animals and during the 6-month period of carcinogen treatment for all animals receiving 2-AAF. The carcinogen was dissolved in ethanol and sprayed onto the meal in a drum mixer. Ethanol was removed by evacuation and the final concentration of 2-AAF was 500 ppm. This feed was given to the mice in a feeder especially designed to prevent spillage so that food consumption could be readily measured. The major endpoints to be examined were the extent of urinary bladder hyperplasia and the presence of bladder tumors. Approximately 30 other tissues were also examined.

The experimental design (Figure 1) may be divided into three major age periods. In each period, the mice were fed 2-AAF at a concentration of 500 ppm for exactly 182 days. This period is represented in Figure 1 by the diagonal shading.

Figure 1



In Period 1, 2-AAF was fed from the start of the experiment for 6 months. In other words, for this period, treatment was started when the animals were 1 month old and terminated when they were 7 months old.

In Period 2, mice were fed 2-AAF for 6 to 12 months into the experiment and they were, therefore, 13 months old when treatment was terminated.

In Period 3, the carcinogen was administered during the experimental period from 12 to 18 months.

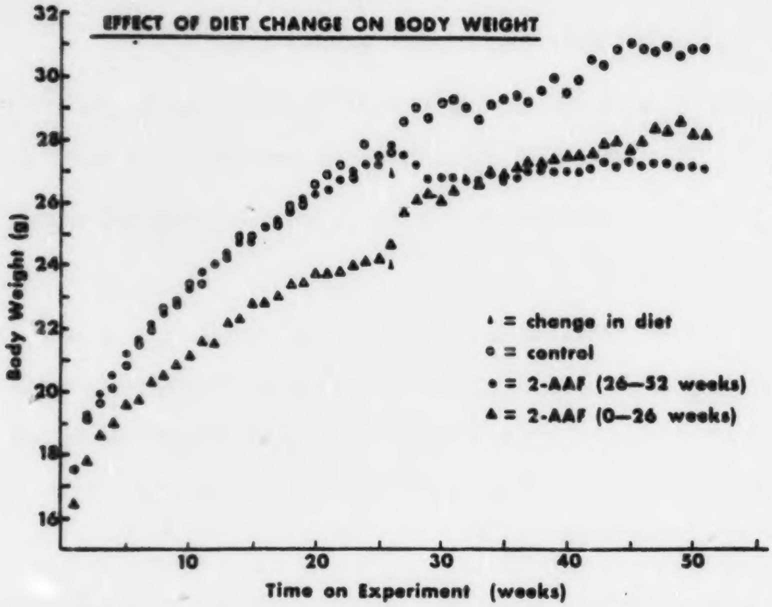
Each of these periods may be further subdivided. One experimental group was killed immediately at the end of the 6-month feeding period. The remaining animals were placed on control rations and one of these groups was killed 3 months later; a second group, 6 months later; and the final group was left on control diet to finish out the remainder of the lifespan.

Control animals were included to match each of the sacrifice times for the 2-AAF treated animals. Three groups of controls were given the meal diet for a 6-month period to correspond with the three periods of 2-AAF treatment. These control groups receiving meal are represented by the cross-hatched bars of Figure 1. The total number of animals on experiment was over 4000.

Data presented here must be considered of a preliminary nature since the experiment is only now at the 18-month stage and data already in hand are still in the process of verification and analysis.

The effect of 2-AAF on body weight can be seen very readily in Figure 2. The rate of weight gain was significantly depressed in weaned animals fed a diet containing 2-AAF. Changing to control feed brought about a rapid increase in body weight but did not completely restore body weight to the control level. Conversely, animals given 2-AAF after 6 months on control diet quickly lost weight, then failed to gain or lose weight during the rest of the treatment period. Weight gain in control animals is indicated by the open circles. During the first 6-month period these animals received pelleted feed. They were fed control meal in the specially designed Center feeder during the second 6-month period. The change in diet form and mode of administration did not significantly alter weight gain in these animals.

Figure 2



Effects of 2-AAF on weight gain may be at least partially explained by changes in feed consumption. In all cases 2-AAF decreased total weekly feed consumption (Table 1). However, this effect of the compound was most striking in the youngest animals and became progressively less apparent as animals aged.

Table 1: Effect of 2-AAF on Feed Consumption in BALB/c Females

Treatment	Average Feed Consumption (g/mouse/week)		
	0-6 mos.	6-12 mos.	12-18 mos.
Control	21.2	21.7	21.1
2-AAF	18.3	19.3	19.9

When feed consumption is expressed relative to body weight (Table 2), food intake is depressed by 2-AAF only in the youngest animals. Even here it should be noted that the amount of food consumed per unit body weight was greatest in the young animals. Thus, the rate of exposure to 2-AAF when expressed in terms relative to body weight was greatest in this group. There was virtually no difference between the two older groups in the rate of their exposure to the carcinogen.

Table 2: Effect of 2-AAF on Feed Consumption in BALB/c Females

Treatment	Average Feed Consumption (g/g body weight/week)		
	0-6 mos.	6-12 mos.	12-18 mos.
Control	0.93	0.73	0.69
2-AAF	0.85	0.72	0.71

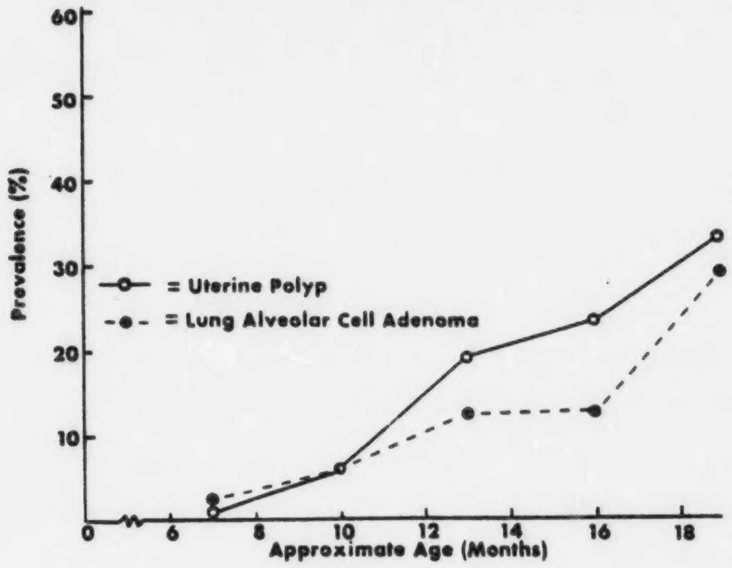
Outside of the effect on body weight, 500 ppm of 2-AAF had little apparent toxicity during the period of treatment. Mortality (Table 3) during the treatment period did not significantly differ from mortality among the control animals. Six to 12 months after termination of 2-AAF treatment, however, mortality of the treated animals is significantly greater than that for the controls. This is especially apparent in the last column of Table 3. To a large degree the higher mortality in these groups can be accounted for by animals dying with urinary bladder carcinomas.

Table 3: Effect of 2-AAF on Mortality in BALB/c Females

Treatment	Time of Exposure	Percent Dead		
		6 mos.	12 mos.	18 mos.
Control	-	1.7	4.7	12.0
2-AAF	0-6 mos.	2.4	6.6	34.3
2-AAF	6-12 mos.	1.8	2.4	20.8
2-AAF	12-18 mos.	1.8	3.0	10.7

The major histopathologic findings of interest in the control animals sacrificed during the course of the experiment are lung alveolar cell adenomas and uterine polyps (Figure 3). Each of these lesions is present in about 30 percent of the control population at 19 months of age. Both of these lesions also have been seen in animals treated with 2-AAF. However, the carcinogen neither seems to increase nor decrease the prevalence of the lesions. To date, of 300 or more control animals that have been examined, only one had bladder tumor and only 2 or 3 animals have exhibited any hyperplasia of the urinary bladder.

Figure 3



PATHOLOGY IN BALB/c FEMALE CONTROLS

Virtually all of the animals killed immediately after 6 months of exposure to 2-AAF exhibited hyperplasia of the urinary bladder (Table 4). This observation is uniform for animals of all three age groups examined. On the other hand, severity of the hyperplastic response is an age-dependent phenomenon. This is demonstrated by the ratio of Grade 1/Grade 2 hyperplasia for each group. Among the youngest animals only one of about three has Grade 2, the more severe grade of hyperplasia. In both groups of older mice, at least 9 of 10 animals have Grade 2 hyperplasia.

Table 4: Bladder Hyperplasia After 6-Month Exposure to 2-AAF

Treatment Period	Hyperplasia		Grade 1/Grade 2
	Prevalence	Percent	
0-6 mos.	158/165	96	1.71
6-12 mos.	208/210	99	0.09
12-18 mos.	62/63	98	0.02

Withdrawal of 2-AAF from the diet results in a partial reversal of hyperplasia (Table 5). Reversal is apparent with respect to the number of animals having hyperplasia as well as in the severity of hyperplasia. In the younger animals, approximately half of the population have lost the hyperplasia 3 months after 2-AAF was removed from the diet. An additional 3 months on control feed has no greater effect. Reversal of hyperplasia in the older animals also appears to have plateaued 3 months after removing the carcinogen. While reversal of the severity of hyperplasia is very striking in this group, a complete loss of the hyperplastic response is seen in only about 10 percent of this population. It will be very interesting to see in what percentage of animals with irreversible hyperplasia, bladder carcinomas eventually develop.

Table 5: Effect of 2-AAF Withdrawal on Bladder Hyperplasia

Treatment Period	Withdrawal Period	Hyperplasia		Grade 1/Grade 2
		Prevalence	Percent	
0-6 mos.	-	158/165	96	1.71
	3 mos.	82/182	45	5.15
	6 mos.	97/196	49	3.00
6-12 mos.	-	208/210	99	0.09
	3 mos.	180/204	88	2.60
	6 mos.	99/110	90	1.33

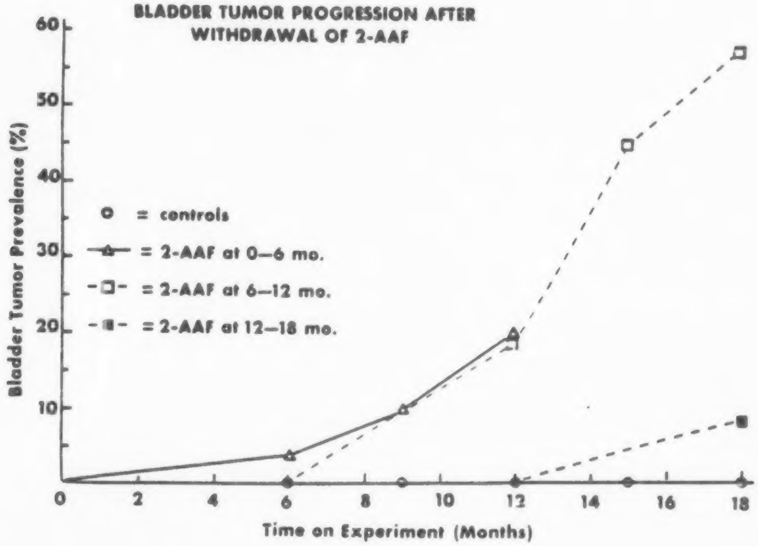
The prevalence of transitional cell carcinomas of the bladder after 6 months of treatment with 2-AAF (Table 6) shows a significant age dependence, both in terms of percent of the population with tumors and the severity of the grade of carcinoma. Animals in the middle-aged group during treatment are apparently most sensitive to the carcinogen. This is true when considering only the zero grade carcinomas, but is especially obvious when the more severe grade of carcinoma is also included. Since only a fraction of the pathology from the oldest aged group has been completed, we can only tentatively say that these animals appear to be intermediate in their sensitivity to the carcinogen.

Table 6: Bladder Neoplasia After 6-Month Exposure to 2-AAF

Treatment Period	Transitional Cell Carcinoma		Grade Distribution	
	Prevalence	Percent	G - 0	G - 1
0-6 mos.	6/165	3.6	6/165	-
6-12 mos.	39/210	18.6	28/210	11/210
12-18 mos.	5/63	7.9	5/63	-

Age differences become even more apparent when examining the progression of tumors after withdrawal of the carcinogen (Figure 4). Progression in the youngest animals during the 6-month period after removing the carcinogen is considerably slower than in the middle-aged group. In the younger group only 20 percent of the population have bladder carcinomas after the 6-month withdrawal period. This contrasts sharply with nearly 60 percent prevalence in the middle-aged population after the same withdrawal period. It is of interest, although perhaps of no significance, that the prevalence of carcinomas in both of these groups is virtually identical at 12 months. One might conclude from this observation that the latent period is, indeed, shorter in the older animal.

Figure 4



In both experimental blocks shown in Table 7, carcinomas appear to progress toward greater severity with time after removing 2-AAF from the diet. Clearly, the number of Grade 1 carcinomas increases with time.

Table 7: Change in Bladder Neoplasia After 2-AAF Withdrawal

Treatment Period	Withdrawal Period	Prevalence of Transitional Cell Carcinoma (Percent of animals killed)				
		G - 0	G - 1	G - 2	G - 3	G - 4
0-6 mos.	-	4	-	-	-	-
	3 mos.	4	4	-	<1	<1
	6 mos.	11	7	1	<1	-
6-12 mos.	-	13	5	-	-	-
	3 mos.	31	13	<1	-	-
	6 mos.	34	23	-	1	-

In conclusion, there are distinct age differences in the sensitivity of BALB/c female mice to the actions of the bladder carcinogen 2-acetylaminofluorene. These age-related differences include differences in the severity and reversibility of bladder hyperplasia as well as differences in the initiation and progression of bladder carcinomas.

QUESTION: You indicated that the latent period for the induction or initiation of the carcinoma and progression differs with age, but you said that the period was shorter in the older animals. Is that correct?

DR. GREENMAN: It was in the middle-aged group.

QUESTION: Do you have any data, or have you evaluated any data, to show why the younger animals in the other age group -- do you attribute this to the immune capacity of the animals or to the lack of initiation and progression of the carcinoma in that age group of animals?

DR. GREENMAN: We have no good indication at this point. On the basis of the difference in response of the two younger groups, one might have suspected differences in immunological capacity. However, results from the older aged animals do not tend to support this suspicion. There may be other aging changes that are involved.

BENCHMARK TOXICITY

Dr. Lawrence Fishbein*

The primary objective of this presentation on benchmark toxicity is to briefly review the objectives and subsequent process of decision formulation of the recent National Center for Toxicological Research (NCTR) - EPA agreement, formulated on May 25, 1975, to determine the feasibility of a benchmark approach to the toxicological evaluation of pesticides by relating pesticide common denominator functional group, physical chemical properties, and spatial configurations to selective toxicological responses.

Three separate tasks will be addressed. Task I stipulates that NCTR will conduct preliminary studies to determine the functional groups, spatial configuration, and/or physical chemical property categories represented in pesticides which are pertinent to predicting common toxicological responses.

Task II is based on results of Task I. NCTR and EPA project officers will select a functional group, spatial configuration, and/or physical chemical property for further study. This will be extensively studied to determine the suitability of data in the open literature and EPA Registration Petition Files for establishing predictive indexes of selective toxicology. A monograph report will be prepared, detailing the state of available information.

Task III deals with benchmark bioassay data. NCTR will conduct targeted bioassays, to produce data of a highly comparable character, which are necessary to support the work of Tasks I and II. The categories to be studied and protocol and schemata will be jointly agreed to by EPA and NCTR project officers.

The process of decision formulation by NCTR will consist initially of a critical examination of pesticide classification by use category, e.g., insecticide, herbicide, or fungicide, chemical classification within the above use categories, and mechanisms of action.

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*National Center for Toxicological Research, Jefferson, Arkansas

Tables 1 through 3 depict the major chemical classifications and subcategories within insecticide, herbicide, and fungicide use categories, respectively. The chemical classes and moieties range from the relatively simple to the complex and offer fertile ground for subsequent, more definitive structure toxicological considerations.

Table 1: Classification of Insecticides

I. Chlorinated Hydrocarbons
  A. DDT analogs
  B. Cyclodiene compounds
  C. Benzene hexachloride

II. Organophosphorus Derivatives
  A. Pyrophosphates and related compounds
  B. Phosphorohalides and cyanides
  C. Dialkylaryl phosphates, phosphorothioates and phosphorodithioates
  D. Trialkyl phosphates and thiophosphates

III. Carbamates
  A. Naphthyl carbamates
  B. Phenyl carbamates
  C. Heterocyclic dimethyl carbamates
  D. Heterocyclic methyl carbamates
  E. Oximes

Table 2: Herbicides

A. Ureas
B. Triazines
C. Carbamates
D. Bipyridinium derivatives
E. Acylated anilides
F. Hydrazides
G. Chlorophenols
H. Nitrophenols
I. Chlorophenoxy acids and esters
J. Arsenical derivatives

Table 3: Fungicides

A. Dithiocarbamates
B. Ethylene bis-dithiocarbamates
C. Benzimadazole derivatives
D. Thiophanates
E. Phthalimides
F. Hexachlorobenzene
G. Chlorophenols
H. Chloronitrobenzenes
I. Oxathiins
J. Dinitrophenyl derivatives
K. Quinones
L. Organomercurials
M. Organotins
N. Antibiotics
O. Cationic surfactant
P. Miscellaneous

There are approximately 10 toxicological responses of major importance that we may choose to evaluate, as shown in Table 4. They are acute toxicity LD_{50}, carcinogenicity, mutagenicity, teratogenicity, cholinesterase inhibition, neurological delayed demyelination, thyroid endocrine, cardiovascular, immunological, hepatotoxic, inhalation toxicity, and induction of lung lesions.

Table 4: Toxicological Responses

A. Acute toxicity (LD_{50})
B. Carcinogenicity-mutagenicity-teratogenicity
C. Cholinesterase inhibition
D. Neurological (nerve demyelination)
E. Behavioral
F. Inhalation toxicity
G. Hepatotoxic
H. Thyroid-endocrine
I. Cardiovascular
J. Immunological

We believe it is important to the initial evaluation of toxicological response of a requisite chemical or structural category within a particular use category, that stress be given to the design of a prototype data collection form which would help collate and possibly simplify the analysis of mammalian toxicity data.

For evaluation of the acute mammalian toxicity LD_{50}, for example, we would stress the 10 salient parameters listed in Table 5. A preliminary assessment of primary and secondary literature sources indicates that there is an amazing, incredible lack of uniformity, standards of excellence, etc. in the obtaining of requisite acute oral LD_{50} information.

Table 5: Acute Mammalian Toxicity

LD_{50} (Dose Lethal to 50% of Animals)

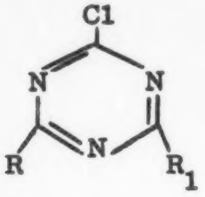
Correlate and Tabulate Information Relative to:

A. Species, strain, sex
B. Age and/or weight
C. Purity of test pesticide (how determined), solvent or vehicle, prep. of solution (ultrasonics)
D. Route of administration and frequency
E. Number of animals used for test and control
F. Duration of observation
G. Method of statistical analysis
H. LD_{50} value (calculated)
I. Site of action
J. Structural formula, stereochemistry (isomers)

Figures 1 through 3 suggest how structure-activity relationships may be considered within one chemical category -- the triazines, for example, of which a number are significant herbicides. These figures depict the substitution, by various alkyl and aryl substituent, of one chlorine at a time, in a basic cyanuric chloride structure, the backbone of the triazines.

Figure 1

Structure-Activity Relationships of Triazine Herbicides: Substitutions of Chlorine Atoms of Parent Cyanuric Chloride



$R = R_1$ = Alkylamine

$R = R_1$ = Alkylamine

R = Alkylamine
R_1 = Dialkylamine

$R = R_1$ = Dialkylamine

R = Alkylamine or Alkoxyalkylamine
R_1 = Alkoxyalkylamine

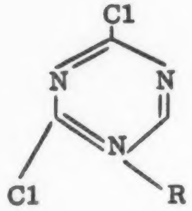
R = Alkylamine
R_1 = Cyanoalkylamine

R = Alkylamine
R_1 = Cycloalkylamine

Figure 2

Structure-Activity Relationships of Triazine Herbicides: Substitutions of Chlorine Atoms of Parent Cyanuric Chloride

Class I: Only one chlorine atom is replaced by one of the mentioned substituents:



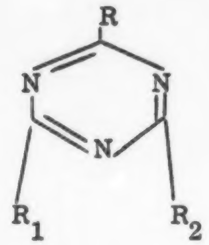
R = 0 - Alkyl
= 0 - Aryl
= S - Alkyl
= S - Aryl

R = NH - Alkyl
Alkyl
= N
Alkyl
= NH - Aryl

Figure 3

Structure-Activity Relationships of Triazine Herbicides: Substitutions of Chlorine Atoms of Parent Cyanuric Chloride

Class III: All three chlorine atoms are substituted:



R = Alkoxy, R_1 & R_2 = Alkylamines

= Alkylthio, R_1 & R_2 = Alkylamines

= Azido, R_1 & R_2 = Alkylamines

= Isocyanato, R_1 & R_2 = Alkylamines

= Alkylthio, R_1 = Alkylamine, R_2 = Azido

R & R_1 & R_2 = Alkylamines

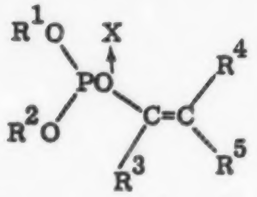
Analogous considerations could be invoked, for example, for the vinyl phosphate insecticides, the basic structure of which is shown in Figure 4. The variation in the group provides a range of not only insecticidal chemical/physical properties, but obviously, of toxicological properties -- the major ones we discussed previously.

We would then, hopefully, correlate the various substituents wherever the literature permits, with observed toxicological response of choice, for example, acute LD_{50} into tabular summaries including the following information: chemical structure, chemical names, alternate names, and relevant LD_{50} information and references.

We are well aware that the number of correlations exist for the correlation of structure activity with toxicological response. These are, for example, the Hansch

Figure 4

Vinyl Phosphate Insecticides



1. R^1 and R^2 are alkyl groups; R^3, R^4, and R^5 can be one of a wide variety of groups including hydrogen, methyl, carboxymethyl, alkylamido, polychlorаryl, etc., X is S or O.
2. The variation in the groups provides the range of insecticidal, chemical, and physical properties that exists with the class.
3. All currently commercially available members have both R^1 and R^2 either methyl or ethyl and all except one have an oxygen atom as X.

Multiple Parameter Method and the Free-Wilson Technique, both of which have been employed with limited success, primarily in the drug industry, in the evaluation for homologous series of compounds, for specific functions such as antimalarial or carcinostatic activity.

We don't believe that there exist sufficient analogous data in pesticide areas of concern at present that would permit the use of these correlative techniques. In summary, we believe that the discussed strategy will permit us to better review and evaluate the requisite data bases. This will allow us to more rationally suggest additional areas of research in order to fill the gaps that our preliminary studies may indicate and, hence, to assist ultimately in the enhancement of the Substitute Chemical Program.

PHARMACOKINETIC MODELING OF SELECT PESTICIDES

Dr. John F. Young*

The use of pharmokinetic parameters obtained from acute studies to predict accumulation of chemicals on subacute or chronic exposure has not yet been attempted in the field of toxicology. The implication that selected pesticides accumulate in various biological systems is not based on pharmacokinetic prediction but on single point determinations. If two animals are from the same chemical environment and one has a higher chemical residue than the other after serial sacrifice, what does this really mean? ... Accumulation? ... Biological variability? ... Different absorption rate constants? ... Different elimination rate constants? ... Different distribution parameters? ... Actually, it is very hard to say unequivocally which is taking place.

However, if we take a small group of animals and determine their pharmacokinetic parameters from a single dose, the extent of biological variation can be determined, and these pharmacokinetic parameters can be used to predict the tissue levels on subacute or chronic exposure to the chemical.

The initial pharmacokinetic parameters are obtained by exposing a small group of animals (four to six) to the chemical by intravenous injection. Several dosage levels are used to determine the effect of the initial amount of the chemical on the pharmacokinetic parameters, if any. Blood, urine, and feces levels of the chemical (and its metabolites) are determined as a function of time. From these data a model is tested by simulating the data with a hybrid computer. When the computer has accurately simulated the actual single animal data, the proper model has been obtained.

In Figure 1 this plot of amount of chemical vs. time illustrates the computer simulation (solid lines) of data (X for amount of chemical in the blood and circles for the accumulative amount of chemical excreted) and the computer prediction of tissue levels (dashed line).

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*Teratology Division, National Center for Toxicological Research, Jefferson, Arkansas

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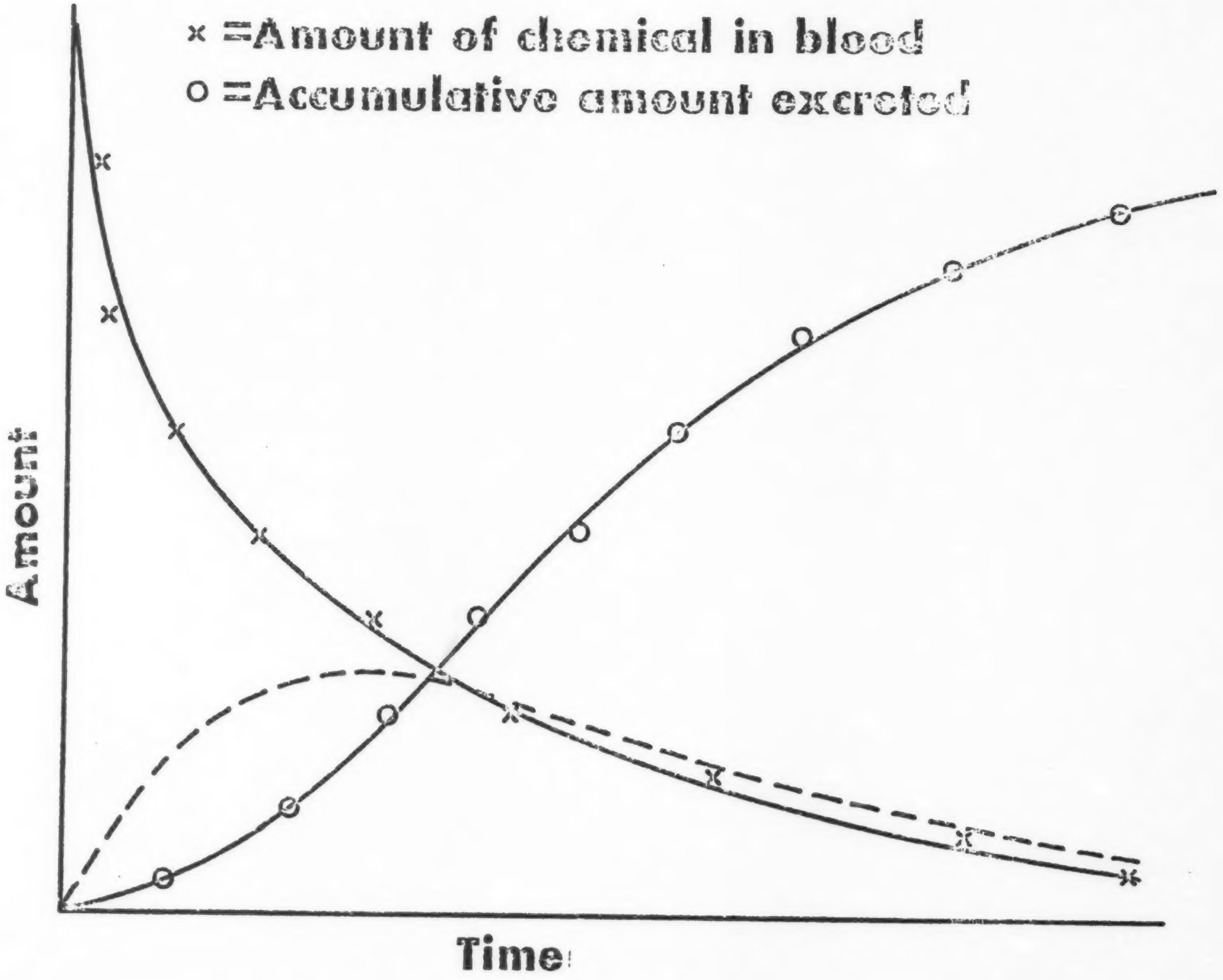
Figure 1



A set of rate constants can be obtained directly from the computer at this time for each animal individually. A simple model might be as shown in Figure 2. This is a two-compartment model with first order elimination. The rate constants are labeled with the first letter representing the compartment which the chemical is coming from and the second letter representing the compartment the chemical is going to. The intravenous information is essential in that it simplifies the model for the most accurate determination of the pharmacokinetic parameters for a given model. However, very few animals are exposed to pesticides by the intravenous route. The next step then is to administer the chemical orally or by inhalation, whichever route has been determined to be the route of environmental exposure. Again blood levels and urine and fecal elimination are analytically determined. On simulating the data with the hybrid computer, the model has now been expanded to include this absorption.

Figure 3 shows a two-compartment model with first order elimination and first order absorption into the blood from "A." We can use these rate constants to determine the biological variability associated with each portion of the model.

Table 1 lists a set of rate constants for four animals with the mean standard deviation, and standard deviation divided by the mean expressed as a percent. These percents are estimates of the biological variation for absorption, distribution, return from the tissue (accumulation), and elimination.

If we utilize these rate constants, we can draw the curves they describe.

Figure 4 is a composite of all four animals treated individually. Each of the sets of curves for A, blood, tissue, and elimination seem to agree fairly closely. It should be pointed out that the half-life for the chemical elimination into the urine is less than 2 days, but the return out of the tissue has a half-life of over 36 days. Which is the proper T1/2 to describe biological half-life?... The return from the tissue is of the most interest for accumulation. If one measures only blood level and does not "model" the data, erroneous conclusions could easily be drawn.

Figure 5 illustrates the accumulation in the tissue for each of the animals of after multidosing at daily intervals with 1/10 the dose of the former curves. The

Table 1: Estimates of Biological Variation

	Absorption	Distribution Accumulation	(Return)	Elimination
Animal	K_{AB}	K_{BT}	K_{TB}	K_{BE}
1	0.316	0.189	0.023	0.436
2	0.295	0.157	0.018	0.387
3	0.310	0.185	0.020	0.400
4	0.280	0.146	0.015	0.360
$\overline{X}$	0.300	0.169	0.019	0.396
S.D.	0.016	0.021	0.003	0.032
$\frac{S.D.}{X}$ %	5.4	12.4	17.7	8.0

Figure 2

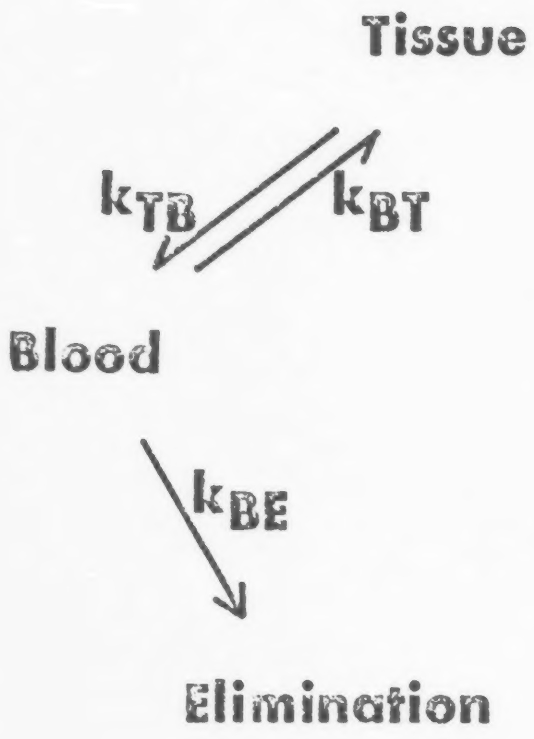

Tissue
k_{TB}
k_{BT}
Blood
k_{BE}
Elimination

Figure 3

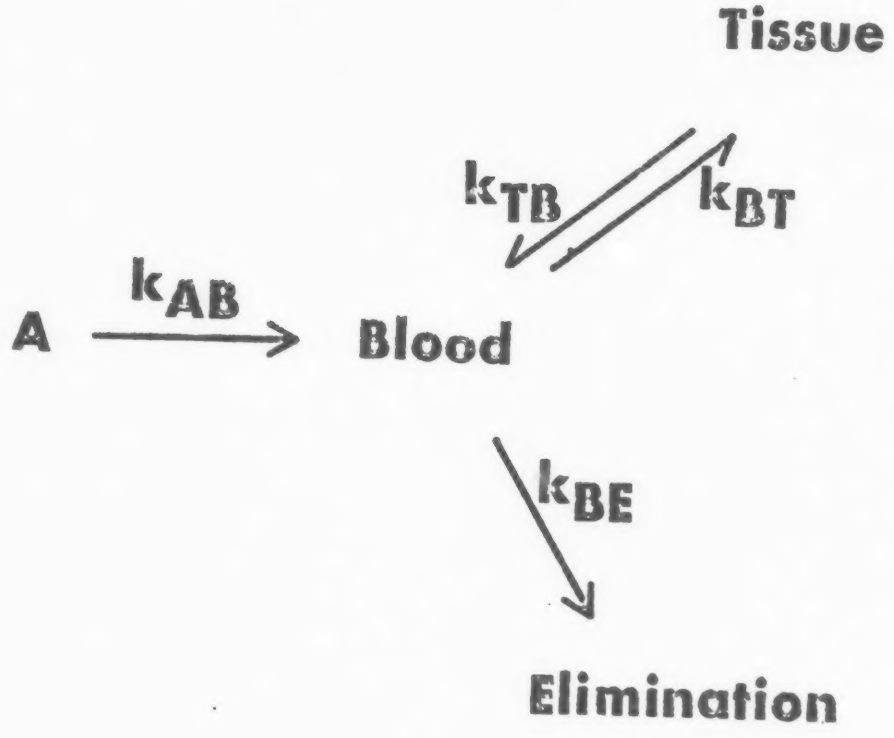

Tissue
k_{TB}
k_{BT}
A
k_{AB}
Blood
k_{BE}
Elimination

Figure 4

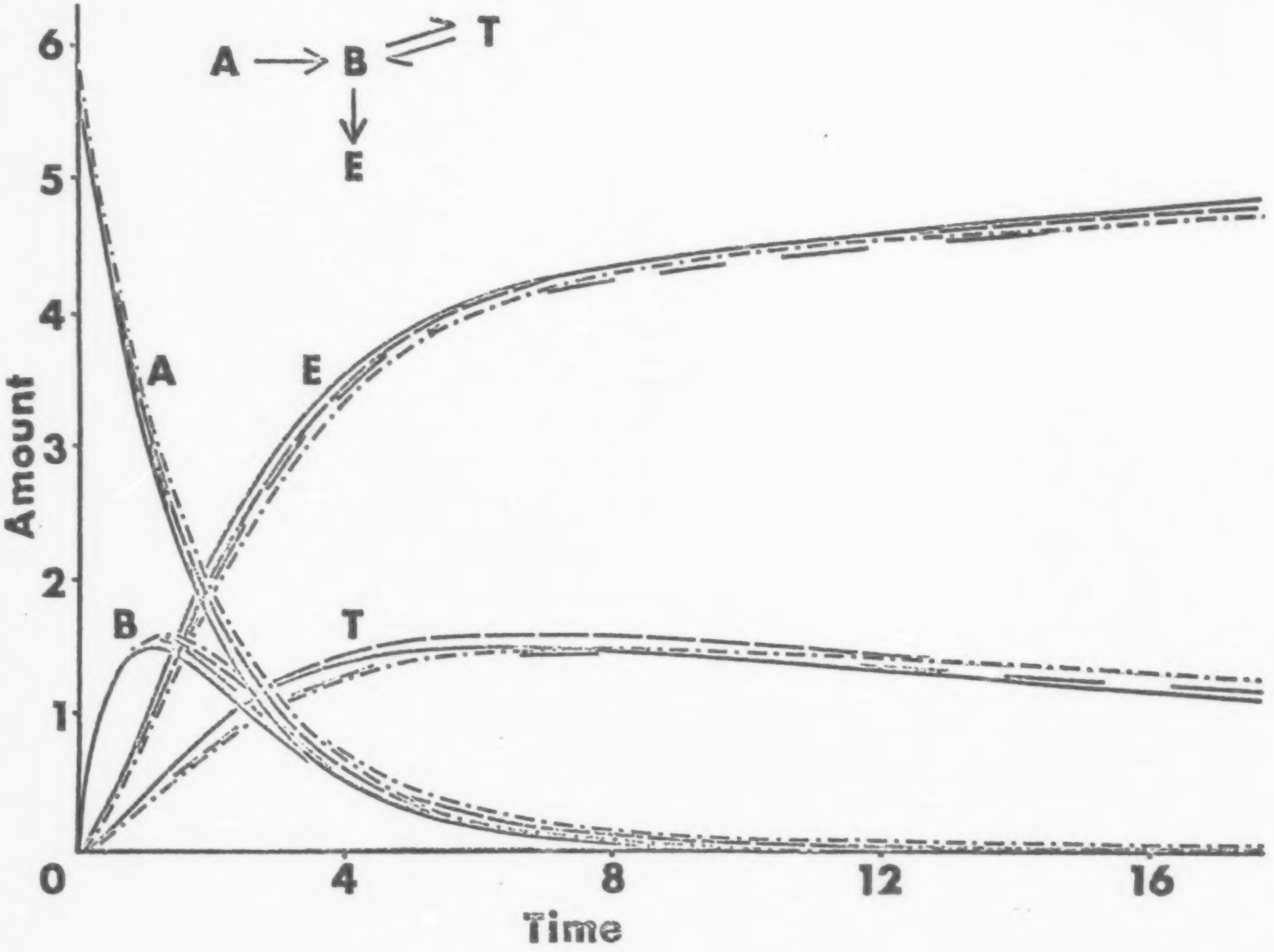
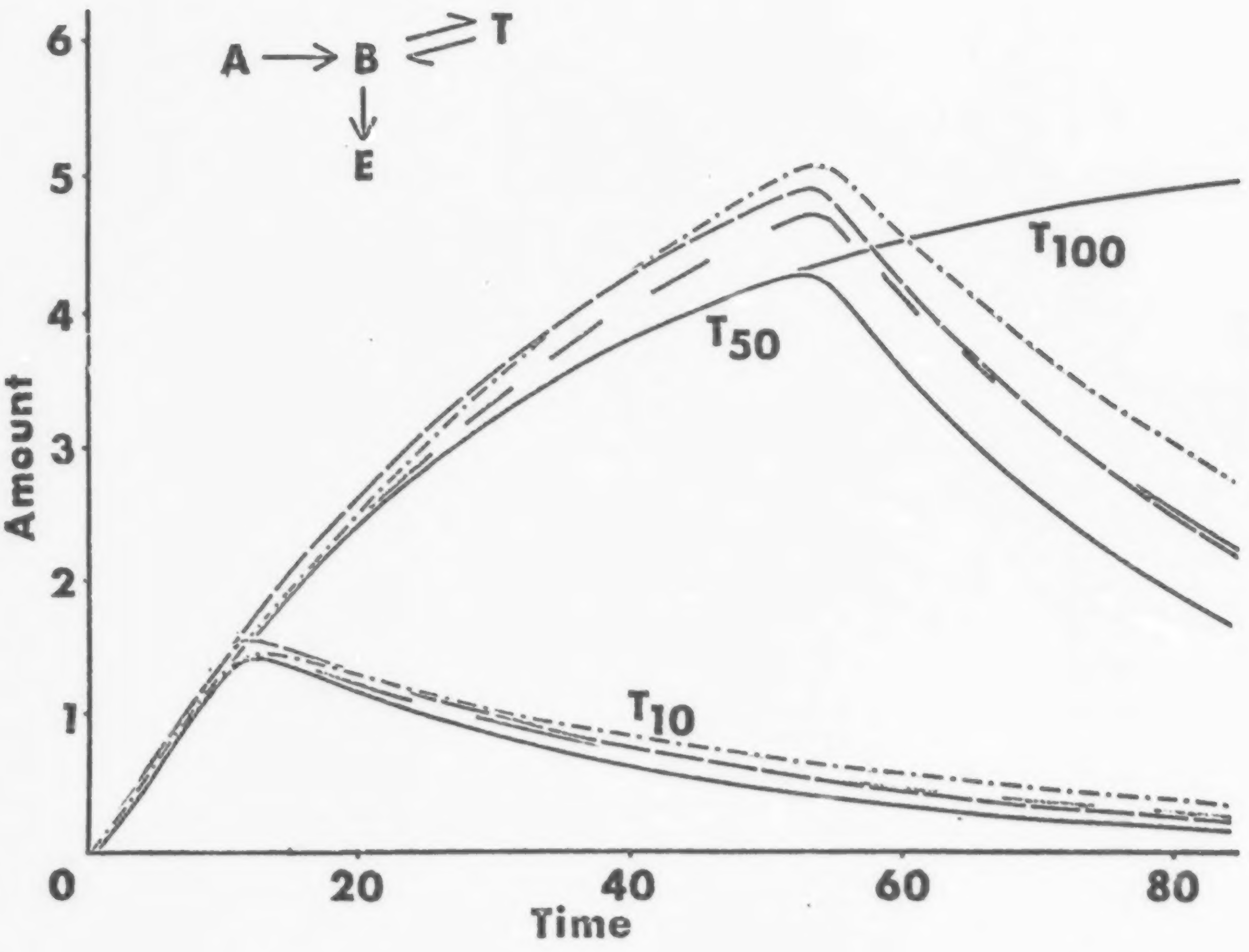


Figure 5



lower set of curves is for 10 doses, the upper curves for 50 doses, and the continued dashed line is for one animal continued for 100 doses. Even after 100 doses, a plateau has not been reached. This particular set of data would not even be classified as a "deep" compartment.

Figure 6 is a plot of amount vs. time showing the blood level, tissue level, and accumulated excretion of the chemical. The lower primed curves are from a single dose and the non-primed curves are after multidosing for 10 doses. The same pharmacokinetic parameters are used for both sets of curves. This is a good illustration of how a seemingly low level of exposure can very quickly accumulate to a fairly high level in the biological system.

If we alter our rate constants to favor the accumulation in the tissue to a greater degree making the tissue represent a deep compartment, the only difference in our pharmacokinetic parameters (Figure 7) is that the return from the tissue has been decreased by a factor of 10. From this it can be seen that a higher level is obtained and maintained for a much longer time period.

The accumulation data on multidosing or multiexposure can be predicted from the single dose exposure studies. This not only provides a decrease in the cost and time involved in such a study, but allows for prediction beyond what would be practical to experimentally determine.

For any new pesticide that is proposed to replace an older established pesticide, the potential to accumulate must be known. The single dose pharmacokinetics and prediction are rapid means by which the old and new pesticide may be evaluated on similar and easily controlled experimental grounds.

Figure 6

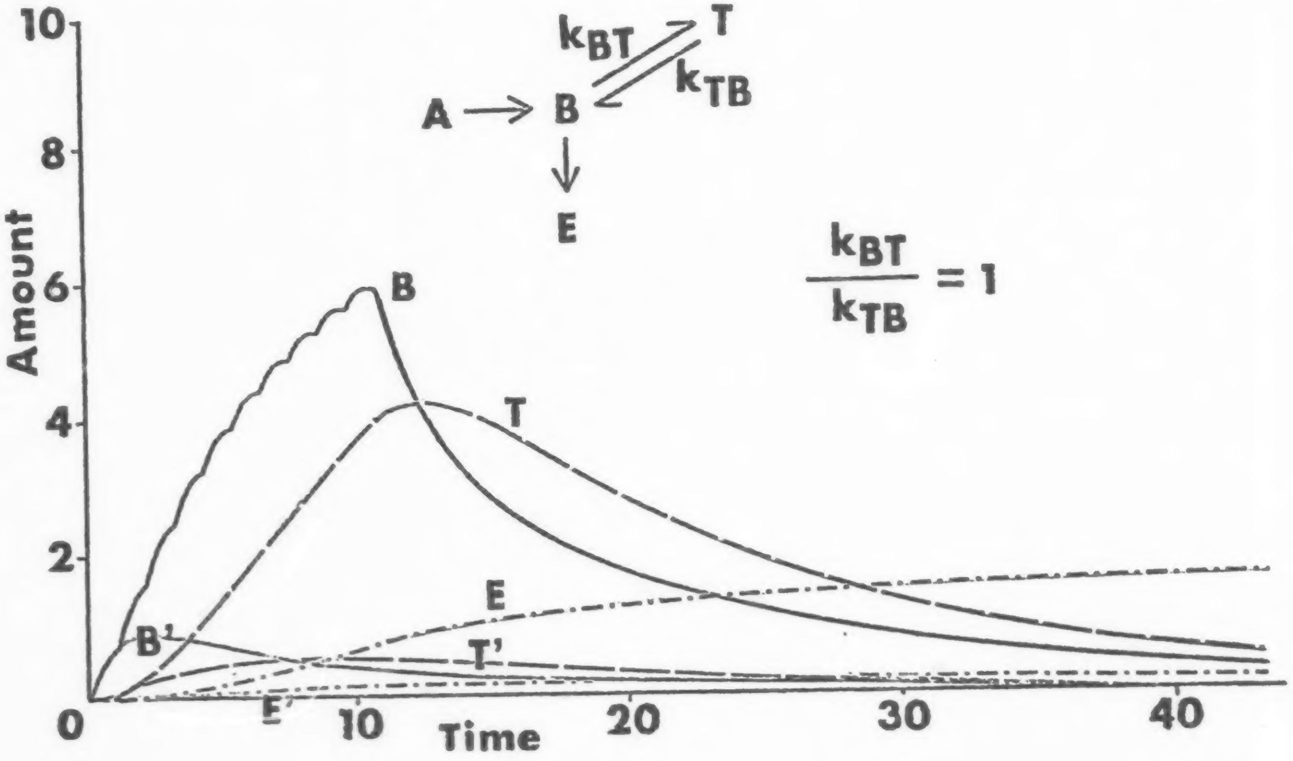
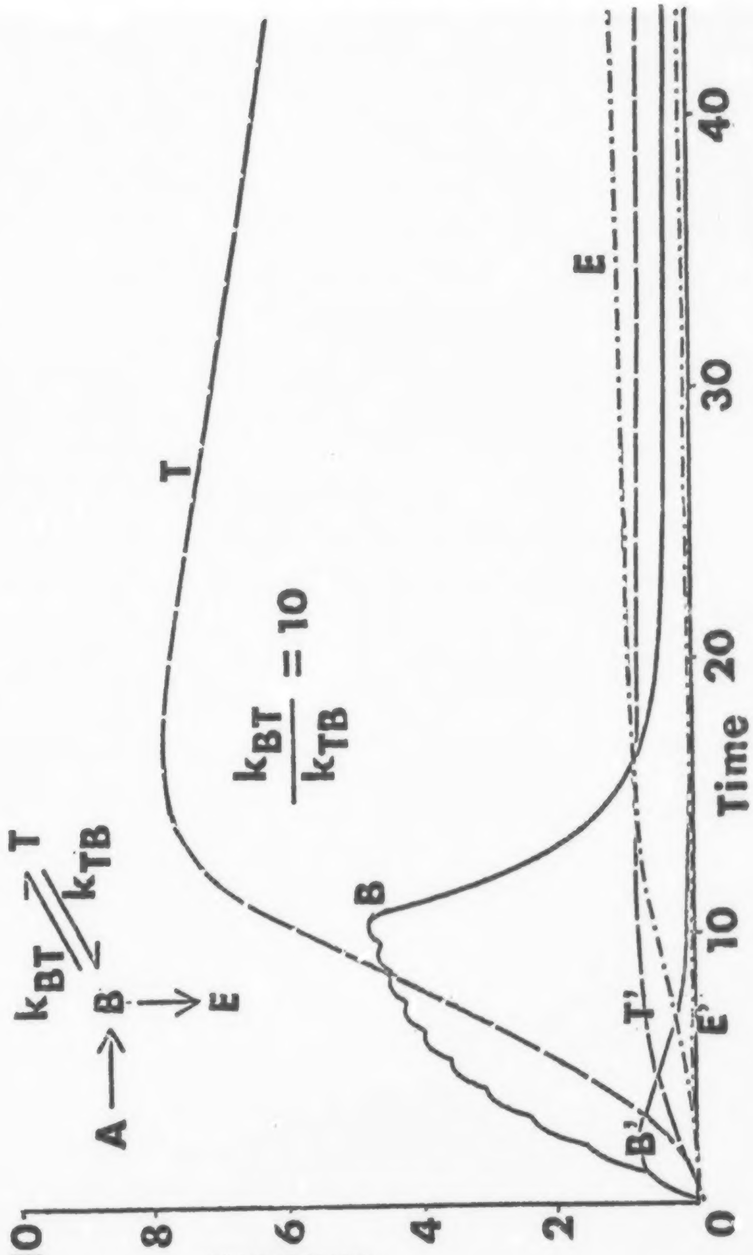

$A \rightarrow B \underset{k_{TB}}{\overset{k_{BT}}{\rightleftharpoons}} T$
$B \rightarrow E$
$\frac{k_{BT}}{k_{TB}} = 1$
Amount
10
8
6
4
2
0
10
20
30
40
Time
B
T
E
B'
T'
E'

Figure 7



$A \longrightarrow B \underset{k_{TB}}{\overset{k_{BT}}{\rightleftharpoons}} T$
$B \dashrightarrow E$
$\frac{k_{BT}}{k_{TB}} = 10$
T
B
T'
B'
E'
E
Amount
Time
0
2
4
6
8
10
0
10
20
30
40

DR. CURLEY: You indicated that the latent period for the induction or initiation of the carcinoma and progression differs with age, but you said that the period was shorter in the older animals. Is that correct?

DR. GREENMAN: It was in the middle-aged group.

DR. CURLEY: Do you attribute this to the immune capacity of the animals or to the lack of development of enzyme systems, or to this lack of initiation and progression of the carcinoma in that age group of animals?

DR. GREENMAN: We have no good indication at this point. On the basis of the difference in response of the two younger groups one might have suspected differences in immunological capacity. However, results from the older aged animals do not tend to support this suspicion. There may be other aging changes that are involved.

PESTICIDE RESIDUES IN HUMAN MILK

Dr. Eldon P. Savage*

The objective of this study is to estimate the levels of organochlorine pesticides in the milk of nursing mothers giving birth in United States hospitals on a nationwide basis. As most of you know, in 1951 Long et al. (1) reported DDT in 30 of 32 human milk studies collected in Washington, D.C. The current study is designed to try to analyze milk samples from 1,600 mothers from across the country so that we get, for the first time, some national levels of organochlorine pesticides in human milk.

The participating groups in this study are Colorado State University, the University of South Carolina Medical School, Mississippi State University, and the two State Health Departments of Michigan and Utah. All of these groups have participated in the community studies on pesticides. They all have laboratories that have been in the laboratory quality control program that is operated by the EPA.

One of the reasons that this study was done is that we had worked up a design for a study on the incidence of acute pesticide poisonings in hospitals across the United States, and that this design is based on the 7,000 plus hospitals that are listed by the American Hospital Association. A sample of the 783 hospitals that were visited in the acute pesticide study was used as a clustering point where we could obtain a list of nursing mothers.

Ideally, what we would obtain would be a list of all the nursing mothers in the United States during a specified time period. Obviously, such a list is not available. Approximately 150 hospitals were randomly selected and these hospitals then became a cluster point for a list of women's names who have given birth in these hospitals during the past year. Then from that list, the number of mothers who are nursing is determined and they are contacted to obtain a sample of milk from a particular group.

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*Colorado Epidemiologic Pesticide Studies Center, Fort Collins, Colorado

Within each pesticide usage level within the five geographical regions, the general hospitals with nursery facilities were stratified according to the number of births in the hospitals for the 1973 calendar year. The sample size selected from a particular hospital is determined by the number of births in the hospital in 1973. The sample size range is from two to three samples per hospital up to approximately 100.

The total number of samples that we thought we could handle in this program, in accord with the amount of monies available, was approximately 1,600. Personnel from Colorado State University covered the Western half of the country, and people from the Michigan State Health Department covered the Northeast. Mississippi State University is covering about five states in the South Central Region and the University of South Carolina is covering the rest of the Southeast.

At the time that the nursing mother is visited, a sample of approximately 10 ml is obtained. At the same time that we are obtaining the milk samples, we are also obtaining information regarding age, the collection point, the occupation of the household head, race characteristics, as well as number of siblings, plus the highest school year completed. In order to gain more knowledge regarding the pesticide usage patterns of the nursing mothers sampled, this study will incorporate a pilot study to survey household pesticide usage. A stratified random sample of 10 percent of the participating nursing mothers will be drawn subsequent to a preliminary analysis of the results of this study. As currently conceived, 5 percent of the participants with the highest levels and 5 percent of the participants with the lowest levels of organochlorine pesticides will be revisited by a field epidemiologist to determine their patterns of pesticide usage. A pesticide usage form will be completed on each household.

Another important aspect of this study is to determine the levels of pesticides in milk from nursing mothers over time. A subsample of approximately 40 women will be drawn in the Colorado Center area and samples will be collected at approximately 7 days, 4 weeks, and 3- to 6-month intervals to determine if pesticides in milk remain constant, increase, or decrease over time.

Analyses in this study will include the following pesticides and metabolites: dieldrin, chlordane, heptachlor, and heptachlor epoxide oxychlordane. When sufficient milk is collected, this milk will be frozen and stored for future analyses for the polychlorinated biphenyls (PCB).

Quality Control

To check the quality of the survey work performed by the nurse participants, 5 percent of the participants will be checked by telephone. This will serve as a check on thoroughness of the nurse in obtaining pertinent data from the study participants. This activity is extremely important since the hospital nurses will receive only the training provided by the field epidemiologist at the time of the initial contact with the hospital.

The study got underway in early April with a training session for the field epidemiologists participating in the study. About 2 weeks later, we had a meeting of the head chemists from the participating laboratories to discuss analytical methodology and quality control. At this time 149 of the hospitals in the United States were contacted, and this includes Alaska and Hawaii. We have completed sampling of 26 percent of the total hospitals. Currently, 19 percent of the 1,600 milk samples are in the laboratory and in some step in the analytical procedures.

Samples have been positive for DDT, BHC, dieldrin, oxychlordane, and HCB. We are also looking for mirex.

The design for analysis has been developed, and we are going to be doing cluster analysis originally, plus chi square, plus some variance analysis.

I doubt very seriously with our time schedule that we will have the follow-up of the 160 mothers on pesticide usage done before early next year.

One interesting thing I probably should say about this particular study is the fact that, prior to starting it, we heard all types of stories about the nursing mother in the United States, that this group nursed and that this group didn't nurse, and that we probably wouldn't be able to get samples from some hospitals. And if there's one thing that we've learned from the hospitals that we've contacted to date, it is that there really is no pattern. Every hospital has been different. In some hospitals we find that practically every woman who gives birth in that hospital is nursing. We can go to another hospital, maybe a fairly large hospital where we need 25 samples, and we find very few nursing mothers.

We have found cooperation to be very good. The one problem that we have had is that some of the hospitals are very busy, and they may not want to participate. When we encounter this, we obtain a list of the women who have given birth, and then by a random design, we contact women from this list.

We hope to have the field work on sample collection done in the next 90 days. And this means that the laboratories that are doing the analysis are really going to be busy. But most of them are geared to where they can run a number of samples in a short period of time. And hopefully we'll have this study completed in the next few months.

QUESTION: Were you trying to relate the source of pesticides in the milk to other exposures?

DR. SAVAGE: We don't have that, based in this study, with the exception of those 150 women whom we'll be following up, the ones who have the highest residues and the ones with the lowest. And we will get some good information on that particular question.

DR. CRANMER: Since the loss of pesticides from the body fat will be a very major contributor in this phenomenon, and you are following up these women, are you making estimates of weight changes or some other index of change in the fat metabolism of the women involved?

DR. SAVAGE: We are not, but we are doing the milk on a fat basis.

DR. CRANMER: Are you following the fat metabolism of the individuals?

DR. SAVAGE: Ideally, we would need something of this nature, but logistically and money-wise, we thought the best we could do was take these 40 women in the Colorado area, and try to follow them over time.

EFFECT OF SUBSTITUTE PESTICIDES ON HORMONE DEPENDENT TISSUE

Dr. Sydney A. Shain*

Our program was initiated about 3 weeks ago; consequently, I will have no data to present. What we would like to do this morning, however, is to present some of the rationale for the design of the study and some of the precedences in the literature on which the study is based. Basically, our program is a pilot study to determine whether or not we can develop a predictive model for the chronic effects of pesticide in steroid hormone-dependent tissue.

The potential of certain pesticides to deleteriously affect reproductive function has long been recognized. In 1950, Burlington and Lindeman (5) reported that DDT caused inhibition of both testicular growth and the development of secondary sex characteristics in cockerels. More recently, Diechmann et al. (6) have observed diminished reproductive activity in male beagles receiving aldrin, which, in part, appeared to be the consequence of a suppression of libido. In addition, Espir et al. (8) have reported the occurrence of impotence among four of a group of five farm workers following exposure to pesticide. These types of observations imply a possible deleterious action of pesticide on accessory organs of reproduction, at extra-organ sites, or a combination of multiple effects at organ and extra-organ sites.

In order to appreciate some of the potential multiple sites of pesticide action, it is necessary to briefly review the mechanism whereby steroid hormones regulate accessory reproductive organ function. A general model for steroid-hormone regulation of cellular function in hormone-dependent tissue has evolved as a consequence of extensive studies with numerous hormone-dependent tissue models (11, 15). This general model, as it applies to the regulation of function in male accessory organs of reproduction, is schematically represented in Figure 1. It is seen that the testicular hormone, testosterone, circulates in the extracellular plasma. In the accessory organs of reproduction, plasma-borne testosterone enters cells by a non-facilitated

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*Southwest Foundation for Research and Education, San Antonio, Texas

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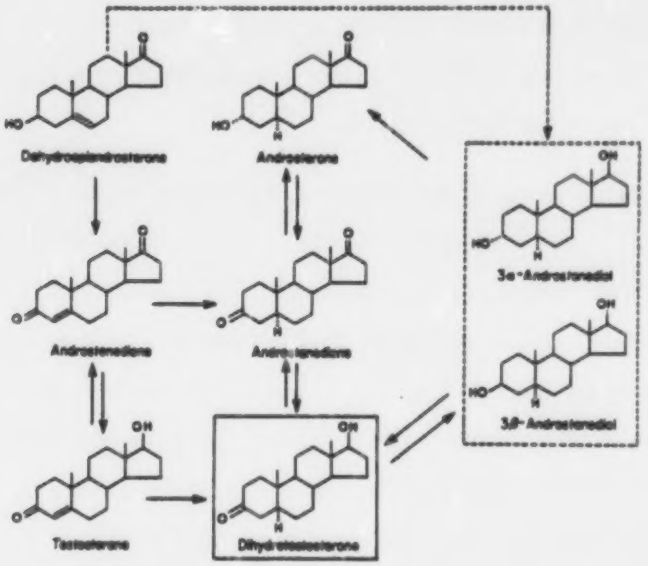
passage across the plasma membrane. Once within the extranuclear space of the hormone-dependent cells, testosterone is rapidly metabolized to the accessory sex organ-specific hormone, 5 α-dihydrotestosterone, by the enzyme Δ^4-3-ketosteroid-5 α-oxidoreductase (4, 14).

Figure 1

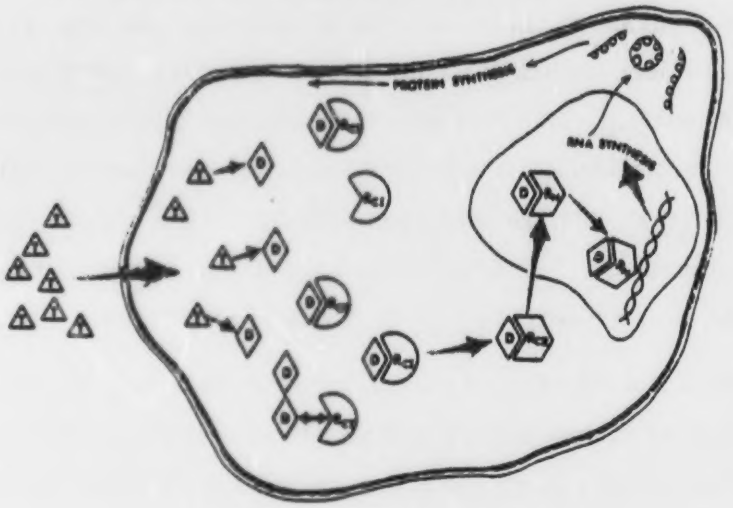
Pathways of Testosterone Metabolism in the Rat Prostate


Dehydroepiandrosterone
Androsterone
3α-Androstanediol
Androstenedione
Androstanedione
3β-Androstanediol
Testosterone
Dihydrotestosterone

Pathways of prostate androgen metabolism are represented in Figure 2. Under normal physiologic conditions, 5 α-dihydrotestosterone is the principal prostatic androgen, whereas the other androgens depicted in Figure 2 are minor components of the total androgen content of the prostate. In the rat ventral prostate, 5 α-dihydrotestosterone concentration is approximately 1.5 times greater than that of testosterone (17). A quantitatively similar distribution of androgens is found in normal canine (9) and human prostate (21). It thus appears that testosterone should be considered to be a prehormone with respect to steroid regulation of male accessory sex organ function.

Figure 2

POSTULATED MECHANISM OF ANDROGEN ACTION IN THE PROSTATE



The next step in the sequence of events (Figure 1) involves the highly steroid-specific (18), high-affinity binding of 5 α-dihydrotestosterone by a class of cytoplasmic macromolecules, the cytoplasmic androgen receptors. These intracellular macromolecules are thought to be the component ultimately responsible for regulation of cellular function. The binding of 5α-dihydrotestosterone by the cytoplasmic receptor, R_{CI}, to form the steroid-hormone complex permits or facilitates a change in receptor conformation with resultant formation of a neonuclear steroid-receptor complex, R_{CII}. As the name implies, the neonuclear steroid-receptor complex is capable of transport across the nuclear membrane. Once within the nucleus, the nuclear steroid-receptor complex binds to highly steroid-receptor complex-specific chromatin sites and subsequently permits the initiation of transcription of DNA sequences into RNA copies which are translated into newly synthesized proteins.

Multiple sites of potential pesticide alteration of androgen regulation of prostate function are apparent. The model implies that prostate competence for androgen is determined by the intracellular content and distribution of androgen receptor. Consequently, an action of pesticide which results in a change in receptor content or distribution would indicate a change in prostate competence with respect to potential androgen regulation of tissue function. Such an alteration in prostate competence could be directly achieved by the binding of pesticide to prostate androgen receptor with the subsequent formation of active or inactive pesticide-receptor complexes. Precedence for this mode of pesticide action exists since dieldrin has been demonstrated to diminish cytoplasmic and nuclear binding of 5 α-dihydrotestosterone in rat ventral prostate preparations (25) and *o,p'*-DDT has been demonstrated to be a specific inhibitor of 5 α-dihydrotestosterone binding to rat ventral prostate cytoplasmic androgen receptor (26). These *in vitro* studies are consistent with the results of acute *in vivo* studies which have demonstrated a diminished accumulation of testosterone by the mouse anterior prostate after exposure to *p,p'*-DDT (22) or dieldrin (24).

A second effect of pesticide at the level of the prostate could be achieved by a change in the capacity of this tissue to metabolize testosterone to 5 α-dihydrotestosterone. Although a number of acute *in vivo* exposure studies have failed to reveal a significant effect of pesticide upon the production of 5 α-dihydrotestosterone by the mouse anterior prostate (7, 23, 24), the addition of pesticide to *in vitro* preparations of mouse anterior prostate has demonstrated parathion reduction (23) and carbaryl augmentation (7) of 5 α-dihydrotestosterone production.

In addition to these possible effects of pesticide at the level of the prostate, it is clear that pesticide can also alter prostate competency by affecting the extraprostatic metabolism of testosterone. The absolute dependence of the young, mature prostate upon peripheral testosterone for the maintenance of normal structure and function is established beyond doubt (16). Consequently, the well-documented capacity of numerous pesticides to increase peripheral metabolism of testosterone by hepatic mixed-function oxidases (1, 7, 10, 12, 24) indicates the potential of these compounds to alter prostate competence. In addition, certain of the organochloride pesticides must be considered to possess the potential to alter peripheral levels of testosterone through changes in the pituitary-gonadal axis as evidenced by the ability of dieldrin to increase serum luteinizing hormone levels in male rats (2). These potential changes in peripheral testosterone levels would be expected to alter prostate androgen-receptor content as evidenced by the observation that orchiectomy of the rat ultimately results in a decrease in the prostatic androgen-receptor content (3, 13) and that the known aging-associated diminution in the androgen-receptor content of the rat ventral prostate (19) may be related to the concomitant aging-associated diminution in plasma testosterone content (20).

We have attempted to indicate the most prominent known potential sites of action of various pesticides which could result in a change in the status of accessory organs of reproduction. Using the prostate as a model for the potential effect of pesticides upon male accessory organs of reproduction, it appears that the ultimate expression of the multiple potential effects of pesticide would be a change in the androgen-receptor status of the prostate. This program attempts to develop the rodent prostate as a predictive model of the potential effects of pesticides upon accessory organs of reproduction and other hormone-dependent tissue. To this end, we will evaluate the effect of chronic ingestion of pesticides upon prostate androgen-receptor levels and distribution and upon selected biochemical parameters which are specific measures of prostate competence. Furthermore, in order to attempt to distinguish extraprostatic effects of pesticide from those which occur at the prostate, we will evaluate plasma-testosterone levels for correlation with the measures of prostate competence.

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DR. CURLEY: In your report, Dr. Shain, I don't think you indicated to us what compounds would be tested. Could you tell us what these compounds are? What the route of administration will be? What the period of administration will be?

DR. SHAIN: We will be using chlordane, heptachlor, methoxychlor, parathion, Diazinon, and carbofuran. The route of administration will be in the diet for a period of 3 months. Each pesticide will be administered to 42 animals and analysis will be on animals in groups of six.

DR. CURLEY: In your introduction you mentioned three studies, one on DDT, one on dieldrin. You mentioned one study that indicated impotence in some group of test animals. Which study was this?

DR. SHAIN: The impotence studies were in 1970 by Espir, and they were in a group of four or five adult male farm workers who had been exposed repeatedly to pesticides and herbicides. The impotence was reversible upon treatment with methyl testosterone. The time required for full recovery -- of course there's a certain amount of psychological recovering necessary -- varied from 4 months to over 12 months.

QUESTION: I think in some in vitro studies with estrogen-dependent tissues, it has been shown that there is a heat-dependent conversion of the estrogen receptor from one form, or one conformation, to another. To your knowledge, has this been shown in androgen-dependent tissues?

DR. SHAIN: The activation step that you're discussing has been demonstrated, as you said, for the estrogen receptor and in addition for the progesterone and glucocorticoid receptor. To my knowledge, there is no similar direct evidence for the prostate androgen receptor, although it too is capable of undergoing some of the transformations that have been shown for the estrogen, progesterone, and glucocorticoid receptors. The general theory would indicate that the androgen receptor would also undergo this transformation; however, the evidence has not yet been presented.

QUESTION: Will you attempt to determine turnover rates of the androgen-receptor complex in this particular study? And also, if you use the Scatchart Plot, will you be able to tell us anything about the site of the binding of that particular compound on the receptor complex? You'll be using labeled compounds, I would assume. Is that correct?

DR. SHAIN: We will not use radioactive pesticides in any of our studies since they are not available in suitable preparations. But, in order to obtain some direct information relevant to the questions which you have asked, we will perform a series of direct in vitro determinations which are designed to evaluate the mechanism of interaction of the pesticides with the receptor proteins. The work of Wakeling has indicated that at least with DDT the interaction may be a noncompetitive type of inhibition.

EFFECTS OF PESTICIDES ON BLOOD LIPOPROTEINS, ARTERIES, AND CARDIAC MUSCLE

Dr. Jack E. Wallace*

This project is under the direction of Dr. Henry C. McGill, Jr., Professor of Pathology at the University of Texas Health Science Center in San Antonio. The information we are presenting consists principally of Dr. McGill's concepts and approach to this problem.

Because of the widespread use of pesticides and the possible exposure of humans to these chemicals over long periods of time, it appears desirable to determine whether or not pesticides in current use, or proposed for future use, affect the cardiovascular system in small doses over long periods of time. In particular, it is desirable to know whether these compounds affect physiological systems and organs in such a way that they may potentiate or accelerate common cardiovascular diseases to which humans are prone.

Arteriosclerosis and its sequelae are the most common cardiovascular diseases in humans in the technically developed countries. The process starts in infancy and develops clinical disease manifestations when individuals reach middle age or later. In this study, we will use the baboon as the model to approximate those conditions which occur in man. When the baboon's diet is made to resemble human diets in the technically developed countries, that is, high in saturated fat and cholesterol, fatty streaks develop in the arterial wall that closely approximate atherosclerotic lesions that occur in humans. If that diet is continued over a long period in the baboon, these fat deposits begin to resemble very closely the lesions that occur in humans.

There are several reasons for using a nonhuman primate for this experiment, and particularly the baboon. The rat is resistant to dietary cholesterol and does not develop atherosclerosis on a high-cholesterol, high-saturated fat diet. The rabbit is just the opposite. That animal is extremely sensitive to dietary fat and cholesterol, much more so than the human. The dog must be subjected to thyroid ablation in addition to being fed an atherogenic diet to develop atherosclerosis. Some species of pigeons develop atherosclerosis spontaneously. Swine also are useful, but are large and difficult to handle. Because atherosclerosis appears to involve many complex interactions, many investigators are turning to nonhuman primates in order to simulate the human situation as closely as possible. The baboon is the largest readily available nonhuman primate, and its sensitivity to an atherogenic diet is very similar to that of the human.

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*The University of Texas Health Science Center, San Antonio, Texas

The Southwest Foundation for Research and Education (SFRE), which is engaged in a joint effort with the Medical School at San Antonio for this study, is well known for its baboon colony. Dr. McGill, the principal investigator, has developed extensive programs of research in atherosclerosis in the recent years involving currently over 400 baboons at SFRE. These animals, the facilities required to house them, and the personnel that are skilled in handling them represent a unique resource.

The baboons are housed in gang cages for breeding and for long-term studies, and in individual cages for studies that require frequent collecting of blood, measuring of blood pressure, or other examinations. The baboon is an extremely intelligent primate, with an elaborate social organization maintained by communicative signals. They respond quickly to training by operant conditioning to perform complex tasks. In one of the projects at SFRE, baboons smoke tobacco cigarettes in a study of cigarette smoking effects of cardiovascular and pulmonary disease. This is accomplished by rewarding them with water when they puff on a lighted cigarette and inhale the smoke.

The diet to which the animals will be subjected along with feeding the respective pesticides will include cholesterol at the level of about one milligram per kilocalorie. Since people in the United States consume on the average about 700 mg of cholesterol per day, or about 0.4 mg of cholesterol per kilocalorie, this diet is similar to that consumed by many humans in cholesterol content. Cholesterol will be derived partly from egg yolk, as in human diets, and the remainder from USP crystalline cholesterol. The dietary fat will be provided by lard at a level of 40 percent of total calories, identical in total fat to the U.S. diet. This same diet is being used in other research projects at SFRE to investigate the effects and mechanism of action of other risk factors on experimental atherosclerosis, such as cigarette smoke inhalation, blood pressure, social stress, and glucose intolerance.

The specific aim of this study is to determine whether pesticides affect the blood lipoproteins that are involved in the pathogenesis of atherosclerosis, or whether they accumulate in the fatty deposits of atherosclerotic lesions of the arterial wall and influence their course, or whether they affect the metabolism of the myocardium. There are several fragmentary reports from the biomedical literature from Russia and Eastern Europe that indicate that they do change the plasma levels of lipoproteins. These lipoproteins, which carry cholesterol, cholesterol esters, triglycerides, and phospholipids in plasma in a stable form, are involved in the pathogenesis of atherosclerosis because certain ones (particularly low density, or β lipoprotein) are elevated in persons who have higher rates of coronary heart disease. It is possible that alterations in the concentrations of lipoproteins, or alterations in their nature, could influence the rate of progression of atherosclerosis. Also, it is possible that the fat-soluble pesticide compounds and their breakdown products may become concentrated in the lipids of the atherosclerotic lesion and influence its course.

The pesticides to be tested are chlordane, parathion, carbofuran, and diazinon. In a preliminary range-finding experiment, we are attempting to find the levels of intake of these four pesticides that will exceed the allowable daily intakes for humans by at least an order of magnitude or more and that will still not produce acute toxic effects. In the definitive study, we will feed each pesticide at two dose levels to six baboons, three males and three females. There also will be a control group that will receive the same diet without added pesticide.

The animals will be studied under basal conditions for one month while on a standard stock diet which is low in fat and cholesterol. Thereafter, they will be placed on the high-fat, high-cholesterol diet without pesticides for 3 months, and their response to the diet will be measured. It is anticipated, on the basis of numerous other experiments with these animals, that the serum cholesterol will rise to about 250-350 mg/dl, which is the range of hyperlipidemia encountered in most humans in the United States. There will be a wide range of variability in these responses to the diet, just as in humans. Among the lipoproteins, the low density lipoprotein (β lipoprotein) will be the principal one to rise.

After the 4 months of baseline studies (one on stock diet, three on the atherogenic diet), the pesticides will be added to the diet in doses to be determined by the results of the range-finding study.

Blood will be drawn at 2-month intervals to determine total serum cholesterol and triglycerides, and at 4-month intervals additional blood will be drawn to perform detailed analyses of the lipoproteins. Lipoproteins will be fractionated by a combination of ultracentrifugal separation and gel electrophoresis. Each lipoprotein fraction will be quantitated with regard to protein content, lipid composition, and pesticide content. For the chlorinated pesticide analyses, we will use electron capture and gas-liquid chromatography. For the carbamates and organophosphates, we will use a thermionic detector.

At autopsy, after 12 months of pesticide feeding, additional samples of blood will be taken for final lipoprotein analyses, all tissues and major organs will be examined for changes that may be related to chronic pesticide administration, and special attention will be given to detailed examination of the arteries to assess the extent, severity, and characteristics of the atherosclerotic lesions produced. Portions of the cardiac muscle will be studied for capability of the mitochondrial fractions to utilize oxygen. We will preserve samples of all organs for possible analysis for pesticides and pesticide residues.

QUESTION: The animal model you are using, as you mentioned, is a very expensive animal model, and it's becoming very limited in supply. When you use inbred baboons, you're even increasing the cost of these studies. We recently did an in-house study at Battelle involving animal model selection, and we found that the tapia, which is a pro-simian, forms these arteriosclerotic plaques and lesions very similar to those of the baboon. I was wondering whether or not your group has looked at any of the pro-simians as a possible alternate animal model.

DR. WALLACE: Feral baboons, not colony bred and reared animals, will be used in this project. Baboons appear to be, at this particular time, the one nonhuman primate that is readily available, and we have had no difficulty in acquiring these animals. We do not have any experience with pro-simians, but others are studying them. The advantage of the baboon is its large size, which makes larger quantities of blood and tissue available.

DR. SPYKER: I'm not entirely clear on your design. It is going to be six animals all together, or six for each compound?

DR. WALLACE: Six for each compound at each dose level and a control group.

QUESTION: Are you analyzing the diet for pesticides?

DR. WALLACE: Yes, the diet is periodically analyzed for pesticides.

QUESTION: What proportion of the baboons may be resistant to the development of atherosclerotic lesions?

DR. WALLACE: There is a wide range of variability in all animal models to the induction of hyperlipidemia and of atherosclerotic lesions. This is also true of the human. There will be some animals resistant to both hyperlipidemia and to atherosclerosis on the diet alone. It is important to determine the effects of pesticides on these animals also.

QUESTION: I may have missed your comment in the design, but what are going to be the relative levels of your pesticide?

DR. WALLACE: These will be ascertained after the range-finding study. The dose will be low enough not to cause acute toxicity, but larger than the allowable daily intake for humans.

DR. FISHBEIN: What is the nature of the pesticides on tests? Are they commercial formulations?

DR. WALLACE: These pesticides will be out of commercial stock as used for pest control.

FETAL TISSUE ANALYSIS FOR PESTICIDE RESIDUES

Dr. Irwin Baumel*

NOT AVAILABLE

FOR

PUBLICATION

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* Metabolic Effects Branch, Criteria and Evaluation Division, Office of Pesticide Programs, EPA

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RESEARCH PROGRESS:
CARCINOGENIC AND TERATOGENIC TESTS - INTRODUCTION

Dr. Morris F. Cranmer*

The compound of the week type of phenomena may very well become compound of the morning and afternoon. As the list grows and grows and grows. For those of you who are not familiar with the list of agents that are under test by the National Cancer Institute, I urge you to examine this list. It may provide some very interesting results to you.

There's also an effort underway, to be coordinated by the National Library of Medicine, to solicit from Government and private laboratories and industry, lists of compounds on chronic bioassay. The purpose of this, of course, is to make this information available at the earliest possible time to anyone who is interested, and to decrease the duplication of these very costly tests that had more than one laboratory. I would hope that we would be successful in bringing an international component into this because, as you all know, there's a great deal of carcinogenic bioassay work which is done overseas. For instance, the British Industrial Biological Research Association does a great deal of this type of work.

There was a public meeting, sponsored by the Toxicology and Related Program Coordinating Committee, on the appropriateness of new mutagenic tests, not only as indicators of mutagenesis, but as indicators of potential carcinogenesis of a compound to be examined. This meeting was successful; it did provide a forum for persons from the Federal Government, universities, and industry to express their opinions on the utility of these techniques. Dr. Gary Flam is chairing a subcommittee which will be preparing a report on the official, if you will, status, as far as DHEW is concerned, with respect to these specific tests.

Looking at the titles to be discussed today I'm sure some of these topics should stimulate some very interesting discussion as to the predictiveness, the utility, and the potential for producing both false positives and false negatives.

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*Director, National Center for Toxicological Research, Jefferson, Arkansas

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IN VITRO AND IN VIVO CARCINOGENIC AND MUTAGENIC SCREEN DEVELOPMENT

Dr. Erling M. Jensen*

The studies being conducted in our laboratories are divided into two general areas of investigation. *In vitro* carcinogenesis assay development studies are being conducted in our Washington Laboratory, and an *in vivo* mutagenesis feasibility study is being conducted in our Worcester, Massachusetts Laboratories.

At the present time, the only absolute methods for determining the carcinogenicity of chemical agents is by *in vivo* bioassays, and even these are sometimes questioned as to their relevance. These assays are extremely time-consuming and expensive, and the need for a simple, reliable, inexpensive, and quantitative assay is obvious. Various microbial assays for mutagenesis or presumptive carcinogenesis fulfill these requirements to a certain extent, but the need for an assay system utilizing mammalian cells still exists.

The ability of chemical agents to transform mammalian cells in culture was first demonstrated by Berwald and Sachs in 1963, and since then has been confirmed by many investigators using a variety of cell cultures. Cells which have undergone transformation in culture exhibit a number of new characteristics, such as a change in colony morphology, loss of contact inhibition, increased growth rate, greater resistance to the toxic effects of the transforming agent, loss of anchor dependence (or growth in soft agar), greater agglutinability by plant lectins, and, of course, ability to produce tumors in a suitable host.

A number of investigators have proposed the use of this system as an assay for carcinogenesis. In each of these assays, colony morphology has been selected as the criterion or endpoint for neoplastic transformation. The assay is illustrated in Figure 1.

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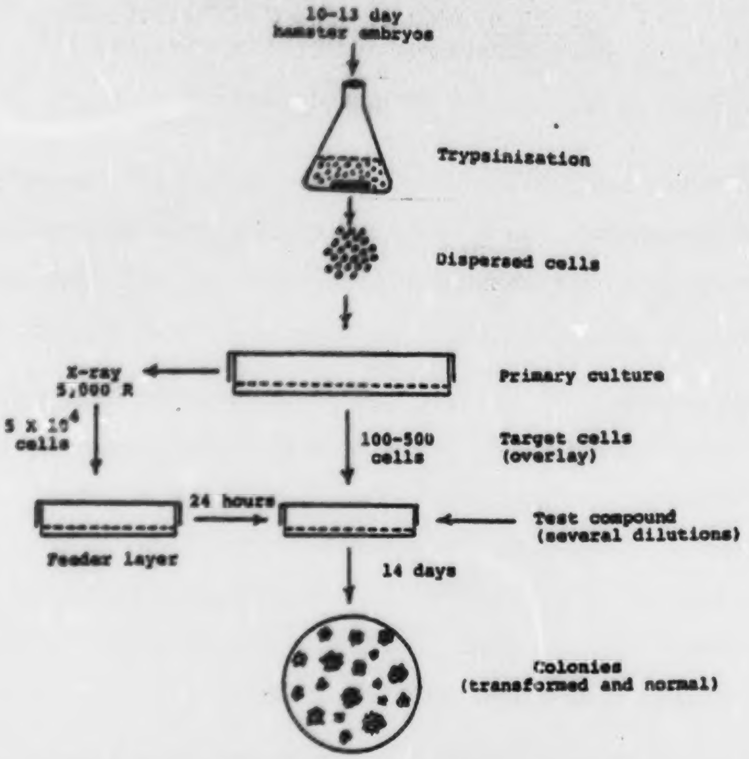
Figure 1

In Vitro Carcinogenesis


10-13 day
hamster embryos
Trypsinization
Dispersed cells
X-ray
5,000 R
Primary culture
5 X 10^4
cells
100-500
cells
Target cells
(overlay)
24 hours
Feeder layer
Test compound
(several dilutions)
14 days
Colonies
(transformed and normal)

Hamster embryos are dispersed by trypsinization to prepare primary cultures which are incubated for several days to eliminate dead cells and erythrocytes. The cultures are then trypsinized to give single cell suspensions from which feeder layer cultures are prepared. Since most mammalian cells grow poorly at low population densities, i.e., 100-500 cells per plate, one can overcome this problem by adding approximately 50,000 X-irradiated cells. This allows the target cells to replicate and form colonies without being overgrown by the feeder cells. After the target cells are added, the test compound is added and the plates are incubated for 9 to 14 days. The colonies can be stained in the culture plates or observed by phase contrast microscopy if one wishes to isolate viable cultures for further study.

Cells which are transformed exhibit a characteristic growth pattern in which the cells tend to pile up through loss of contact inhibition and grow in a disorganized, criss-cross pattern. The neoplastic nature of this type of colony has been confirmed by *in vivo* transplantation, and it is rarely seen in untreated control cultures.

Table 1 shows the results of one experiment with 4-aminobiphenyl in which a relatively high rate of transformation was observed, even though a good dose response was not achieved. The cloning efficiency data, based on an initial inoculum of 500 cells per plate, show a slight toxic effect at 1.2 μg/ml.

Table 1: Transformation of Hamster Embryo Cells by 4-Aminobiphenyl

Compound Conc.	Average Colonies/Plate	Cloning Efficiency	Transformed/ Total	Transformation Rate
Control	52	10%	$\frac{0}{542}$	0%
1.2 μg/ml	33	7%	$\frac{7}{423}$	1.65%
0.6 μg/ml	47	9%	$\frac{2}{471}$	0.4%
0.3 μg/ml	53	10%	$\frac{28}{778}$	3.6%

Therefore, this system can be used to demonstrate neoplastic transformation by chemical agents. However, the assay is extremely sensitive to any variations in the culture environment, such as the type and age of cells used, culture medium, temperature, pH, trace nutrients, and method and time of treatment. Many months of laboratory experience are required to develop reliable assay results. We feel that we now have a very clear understanding of this assay system, and in a very few months we should be ready to apply it to the testing of selected pesticides.

This assay has considerable potential as a qualitative screen for carcinogenesis, but the standard endpoint of morphological alterations is quite subjective and, because of the low incidence of transformation, truly quantitative results are difficult to achieve. Therefore, we are investigating another endpoint based upon the ability of transformed cells to grow and produce colonies in suspension in soft agar. For reasons which are unknown, most normal, untransformed cells require a surface of some type to attach to before mitosis can occur. Most cancer cells and virus-transformed cells, on the other hand, become "anchor independent" and can grow and produce sizable colonies in soft agar. If this phenomenon is true also for chemically transformed cells, a very rapid, quantitative, sensitive, and precise assay for carcinogenesis can be developed, comparable to the microbial mutagenesis assays.

Our studies in this area have been devoted primarily to establishing the reliability of the phenomenon as an indicator of malignancy and to establishing the culture conditions which are required for such growth. We have examined a number of freshly isolated tumor cell cultures, established transformed cell lines, and normal untransformed cultures.

Table 2 shows our results to date. As the data show, growth in soft agar correlates well with the neoplastic or transformed state of the cells, although the percentages of cells forming colonies, or colony-forming efficiency, varies considerably in each line. Two of the human cancer lines have not produced colonies, and we are now attempting to determine if this is a problem related to cultural conditions or to the cell line itself. Our goal is to demonstrate that chemically transformed cells will also grow consistently in soft agar, and these studies are now in progress.

Table 2: Growth of Cell Cultures in Soft Agar

Cell Cultures	Type	Colony Forming Efficiency
B-16, Melanoma	Cancer	40%
Lewis Lung Carcinoma	Cancer	10%
R-35 Mammary Adenocarcinoma	Cancer	5%
13762 Mammary Adenocarcinoma	Cancer	5%
P-388 Leukemia	Cancer	40%
XC, Rat Carcinoma	Cancer	> 90%
HeLa Cervical Carcinoma	Cancer	10%
MDA-MB-134, Breast Cancer	Cancer	0%
MDA-MB-157, Breast Cancer	Cancer	0%
MDA-MB-231, Breast Cancer	Cancer	6%
FL, Human Amnion	Transformed Cell Line	37%
WI-38	Untransformed Cell Line	0%
BALB/3T3, Mouse Embryo	Untransformed Cell Line	0%
Primary Hamster Embryo	Untransformed Culture	0%

The *in vivo* mutagenesis program being conducted in our Worcester Laboratories is a feasibility study to determine whether broad phenotypic parameters, such as behavioral, biochemical, and/or physical characteristics, which are controlled by multiple genetic loci, can be used for detecting mutagenic effects of chemical agents. If this is shown to be possible and practical, an assay can be developed with a much greater probability of detecting mutagenic agents than is now possible with the established *in vivo* specific locus assays.

The experimental plan for this program is illustrated in Figure 2. The assay requires approximately 14 months for completion. Because of the high level of effort required in the final testing phase, new compounds are introduced at 2-week intervals. Briefly, groups of six male C57BL/6J mice are treated with known mutagenic agents at three dose levels below the maximum tolerated dose. Treatment is administered daily for a period of 8 weeks to assure maximum exposure of the germ cells to the mutagen during all phases of spermatogenesis. The treated males are mated, and subsequent breeding is conducted by the cross-backcross method, which is the most effective method for accumulation of homozygous genotypes of recessive mutations. In this process, F_2 females are mated back to their F_1 sires.

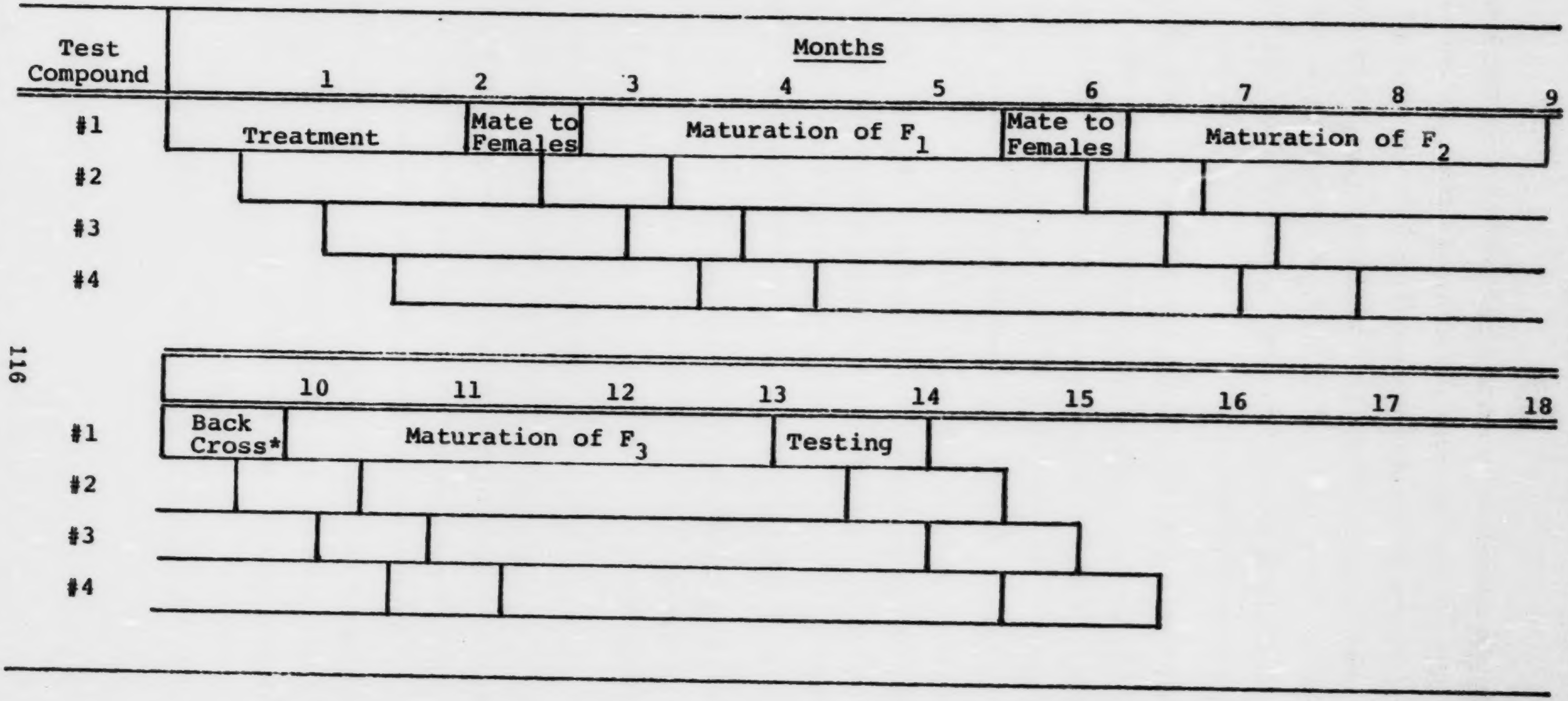
Some testing is conducted on the F_1 progeny for the detection of dominant mutations. Most of the testing will be conducted on the F_3 generation to detect both dominant and recessive changes.

Three behavioral tests are being employed in this study: shuttling activity, active avoidance acquisition, and Go, No-Go discrimination. These tests were chosen because variation in the genotype is known to cause changes in these behaviors, because they can be automated assuring objective measurements and allowing mass screening with few technicians, and because they make use of the same testing apparatus and support systems. Each test measures separate behavioral domains which involve vision, hearing, general arousal, acquisition of new habits, inhibition of learned habits, and intermodal stimulus discrimination.

Figure 2

In Vitro **Mutagenesis**

Experimental Plan


Test Compound
Months
1
2
3
4
5
6
7
8
9
#1
Treatment
Mate to Females
Maturation of F_1
Mate to Females
Maturation of F_2
#2
#3
#4
10
11
12
13
14
15
16
17
18
#1
Back Cross*
Maturation of F_3
Testing
#2
#3
#4

* Mate females back to sires.

The C57BL/6J mouse was selected for these studies because it is a standard strain used in many behavioral genetic analyses, and there already exists a body of data on its behavioral characteristics which can be used in mutagenic studies. Base line behavioral studies are being conducted on C57BL/6J and DBA/2 mice and their reciprocal F_2 hybrids. These studies indicate that this system is capable of detecting genetic variations or mutagenic effects with as few as 70 mice per test group.

Other endpoints will be observed as indicators of mutagenic effects, including general phenotypic abnormalities, such as skeletal deformities, pre-weaning mortality and juvenile death, litter size, anemia at birth, sterility, and alteration in glucose metabolism.

Ten known mutagenic agents are being examined by this procedure. Table 3 shows the current status of the study and some of the problems which have been encountered. These initial studies were conducted at one subtoxic dose level. Unfortunately, almost all published toxicity data on these compounds describe single-dose treatment regimens with essentially no information concerning continuous treatment such as we have employed. There was, therefore, no way of anticipating the delayed mortality and sterility which we experienced initially with some of the compounds. For example, all of the mice treated with N-nitroso-diethylamine died after termination of the treatment, some as much as 3 months after treatment. Several other treatment groups have been unable to breed after either 1 or 2 months. Compounds which have shown these problems are being retested at several lower dose levels. However, six treatment groups are breeding, several of which have reached the backcross stage, and final testing results will be available in approximately 4 months.

This is, as I mentioned, a feasibility study to determine whether and how consistently mutagens can be detected by this approach. Should it prove reliable, a specific assay procedure utilizing the optimal treatment procedures, endpoint determinations, and minimum number of mice will be established for the assay of pesticides and other agents.

Table 3: Current Status of In Vivo Mutagenesis Assay

	Compound	Dose (mg/kg/day)	Route	Status
1.	N-Nitrosodiethylamine	3	Gavage	Delayed deaths
2.	N-Nitroso-N-methylurethane	8	Gavage	F_2 progeny - back crossed
3.	3-Methylcholanthrene	25	Gavage	Some sterility and deaths in F_1, some F_2 progeny
4.	Nitrogen Mustard, HCl	10	S.C.	Delayed deaths and sterility
5.	Methylmethanesulfonate	37.5	Gavage	Sterility
6.	Ethylmethanesulfonate	25	Gavage	Some sterility, some F_2 progeny - back crossed
7.	7, 12-Dimethylbenzanthracene	0.1	S.C.	Delayed deaths
8.	Streptozotocin	0.1	I.P.	F_2 progeny
9.	Tolazotoluidine	50	Gavage	F_2 progeny
10.	4-Aminobiphenyl	30	Gavage	F_1 progeny

DROSOPHILA MUTAGENESIS TESTS

Dr. Ruby Allen Valencia*

The WARF Institute contract with EPA calls for screening for germ line mutations, i.e., molecular alterations in genes or structural changes in chromosomes, which occur in the germ cells and therefore affect the descendents of the exposed individual. Our purpose is to look for mutations in a eukaryote, where there is a digestive mechanism, and other enzyme systems, such as microsomal enzymes, which could convert the compound in question to a different form, resulting in either a loss of genetic effectiveness or the acquisition of mutagenicity.

Our test organism is Drosophila melanogaster, the fruit fly. Drosophila is well known as a tool for genetic studies. It has many genetic and economic advantages and since these are also well known, I will not describe them here. As a screening organism for environmental mutagens, however, Drosophila used to be considered to be at a disadvantage by not being a mammal and therefore too different from man with regard to the physiological barrier between the chemical and the genes. It has been found, however, that flies do indeed have a microsomal enzyme system, eventhough they don't have livers. It has been shown that mutations are obtained with substances known to require enzymatic activation (in the Salmonella test, for example). This work was done by Vogel and Sobels and is in press (1). Thus we now feel on much safer ground with Drosophila testing, and this is most fortunate, since there are no other eukaryotic systems that offer the versatility of Drosophila.

Our lab was installed just a year ago and testing was started in the fall. At the end of the year it was decided that our program would be intensified, and this involved further expansion, hiring and training of additional technicians, etc. We have, therefore, been in full operation only since about February of 1975. We have been sent 20 compounds in the course of the year and we have run them all through our basic pre-determined screening procedures.

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We were lucky that the interest was in looking for effects from low level concentrations, since many of the compounds are insecticides and are, of course, lethal to flies. They can be applied only at very low concentrations. The LC_{50} for some was 0.1-1.0 ppm so we treated at below that concentration. The non-toxic compounds (most of the fungicides and herbicides) were used at concentrations of 2 to 4 ppm.

Exposure was by feeding. The chemicals are dissolved in DMSO, then fed in 1 percent glucose solution on filter paper discs. Actually, exposure is by both ingestion and contact, since the flies must rest on the wet filter paper to feed. Also, the treatment chambers are quite small, so it is difficult for them to avoid contact. We expose for 48 hours, which insures ingestion, since flies cannot survive that long without taking liquid.

The original plan involved two genetic schemes, one for sex-linked recessive lethals and one for chromosomal alterations. These schemes are diagrammed in Figures 1 and 2. In the sex-linked recessive lethal test we score for any mutation of any of about 200 genes on the X chromosome, which results in lethality. In the chromosomal mutation test we score for chromosome breakage, rearrangement, loss, or non-disjunction.

At mid-year when we intensified the program, we added dominant lethals, since these data can be obtained much faster and easier in Drosophila than in the mouse, and they should be just as meaningful. A flow plan was set up, whereby we would get dominant lethal data in 10 days after treatment (that is for the entire germ cell development period), chromosome mutation data in 2 1/2 to 3 weeks, and recessive lethal data in 6 weeks. The dominant lethal test, however, took quite a while to develop and meanwhile most of the chemicals had been tested with the other schemes. It seemed unnecessary to go back and do dominant lethals for these compounds. Also, there is beginning to be a consensus that dominant lethals are not a suitable screening system, especially in Drosophila. Vogel and Sobels (1)

Figure 1

Genetic Scheme for Detection of Sex-Linked Recessive Lethal Mutations

P_1	3 $\frac{\text{FM6}^{(1)}}{\text{FM6}^{(1)}}$ ♀♀	X	1 $\frac{\text{CS}^{(2)}}{\text{Y}}$ ♂ (treated)
F_1	1 $\frac{\text{FM6}}{\text{CS}}$ indiv. ♀ Daughters of each P_1 ♂ kept as a "family" group	X	2 $\frac{\text{FM6}}{\text{Y}}$ ♂♂
F_2	$\frac{\text{FM6}}{\text{FM6}}$ $\frac{\text{FM6}}{\text{CS}}$ X $\frac{\text{FM6}}{\text{Y}}$ When no CS ♂♂ are present, indicating a lethal, make this mating		$\frac{\text{CS}}{\text{Y}}$ Observe cultures for presence or absence of these ♂♂
F_3		Observe for confirmation of lethals	

(1) FM6 = First Multiple No. 6, an X-chromosome with a complex of inversions (to suppress crossing-over) and visible markers (y B w)

(2) Canton S wild-type stock

Figure 2

Scheme for Chromosome "Mutations"

P_1	$3\ \frac{y\ v}{y\ v}\ \frac{vg\ bw}{vg\ bw}$ ♀♀ (1)	X	$\frac{y\ cho}{y^{+}\cdot Y\cdot B^{S}}$ ♂ (2) (treated)		
	Regular Classes	**Exceptional Classes**			
F_1	$\underline{y\ v}$ $\underline{y\ v}$ $\underline{y^{+}.Y.B^{S}}$	$\underline{y\ v}$ $\underline{y\ v}$ Loss of X or Y	$\underline{y\ v}$ $\underline{y\ v}$ $\underline{y^{+}.Y-}$ Loss of B^{S}	$\underline{y\ v}$ $\underline{y\ v}$ $\underline{-Y.B^{S}}$ Loss of y^{+}	Also: 3 X 3 N Mosaics
F_1	$\underline{y\ cho}$ (no Y)	$\underline{y\ cho}$ $\underline{y^{+}.Y.B^{S}}$ Non-disjunction in ♂	$\underline{y\ cho.Y^{L}B^{S}}$ Product of X;Y exchange		Also: 3N "♂" intersexes Mosaics

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(1) The X chromosomes are attached and carry the visible markers "yellow" (body color) and "vermilion" (eye color). The second chromosome carries "vestigial" (wings) and "brown" (eye color) to indicate non-virginity of P_1 females.

(2) The X carries "yellow" and "chocolate" (eye color). The Y is marked on one tip with the dominant normal (grey) allele of yellow and on the other tip with Stone's Bar (a very narrow eye).

(3) All F_1 classes are heterozygous for vg and bw.

have shown this in comparative studies. Therefore we have not been doing the dominant lethal test, although we did work on it and got it going.

The chromosome mutation test also did not rate well in the Vogel and Sobels experiments, as compared to the recessive lethal test. We have applied the test, however, to all 20 compounds and we have at least preliminary data for all of them.

The sex-linked recessive lethal test was found (also by Vogel and Sobels) to be far more sensitive than either of the other two tests. It is capable of indicating mutagenicity at much lower concentrations of the chemical and it works for a much wider range of types of compounds. In view of this, I feel that we should perhaps concentrate our efforts on the recessive lethal test in future testing.

We treat adult males of the appropriate genotype for each type of test, and these males are then mated and brooded sequentially, so that we sample germ cells exposed at different stages of development. In each test scheme, individual treated males are followed, so that we can detect clustering (i.e., the occurrence of two or more mutations in the germ cells of one male). This procedure allows conclusions to be drawn from a much smaller sample size than would otherwise be necessary. I will point out examples later.

We usually treat a batch of flies every other week. When we do, we set up both kinds of tests and we apply two or three different compounds plus one control for each test scheme.

The results from the recessive lethal tests are as follows:

Table 1, for Guthion, shows how the data are compiled. In this case, there were two "runs," each with its parallel control. The table shows the number of treated or untreated parental males, the number of F_1 daughters tested in each sequential brood, and the number found to carry lethal-bearing X chromosomes. Lethals occurring in clusters are so indicated. The standard errors are calculated using

Table 1: Recessive Lethal Data

Guthion

Compound	No. P_1 Males (1)	No. F_1 tests per stage [1/N] (2)				Total F_1 Tests	Total Lethals			% ± se
		1	2	3	4		S (3)	Cl (4)	T	
#1	15	0/1201	1/748	1/398	1/347	2697	1	1(2)	3	0.111 ± 0.083
#2	29	0/1561	0/728	0/453	4/670	3416	4	0	4	0.117 ± 0.058
Σ 7/17/75	44	0/2762	1/1476	1/851	5/1017	6113	7	0	7	0.115 ± 0.043
Control #1	10	0/783	0/104	1/317	1/178	1384	2	0	2	0.144 ± 0.102
Control #2	29	2/1272	2/661	0/290	0/610	2837	4	0	4	0.141 ± 0.070
Σ 7/17/75	39	2/2055	2/765	1/607	1/788	4221	6	0	6	0.142 ± 0.058

(1) No. of treated or control males which produced progeny
(2) No. of F_1 females which produced progeny (lethal bearing/normal)
(3) Single lethal in progeny of one male
(4) Cluster of lethals in progeny of one male (no. of clusters/size of cluster)

the standard formula when there are no clusters. When clusters are present, we use a formula devised by H. J. Muller, which corrects for the probability that the lethals of a cluster are of common origin. The effect is a larger error and avoids attaching undue significance to an increased number of lethals.

Table 2 shows the data from all the control runs. It is generally consistent and the overall frequency (0.18 percent) is within the usual range for this stock (wild type Canton S). Run No. 3 was a bit high and No. 12, our last run, was very much out of line. I will come back to this run later and discuss it further.

Table 3 shows the sum of all results for each compound. With the standard errors derived from the Muller formula, none of the frequencies is significantly greater than the overall control frequency.

Malathion gave the highest overall frequency. The cluster information, however, shows that 21 of the 31 lethals were found in the progeny of a single male. These lethals were found in all broods of that male and certainly represent a cluster arising from a single spontaneous event in a gonial cell. Assuming this to be the case and counting only 11 total lethals, the frequency becomes 0.16 percent. Even counting the 31 lethals, the standard error derived from the Muller formula removes the significance of the higher percent. This case shows the importance of following individual treated males and recording clusters. Table 4 shows the malathion data in detail. Run No. 1 gave a frequency of 0.963 percent versus a control of 0.144 percent. This would have been read as a strong positive mutagenic effect in a standard mass-mating type lethal test. The repeat run would have disproved this conclusion, however, thus demonstrating the importance of repeat runs.

Table 5 shows the bromacil data. In Run No. 1 bromacil gave an elevated frequency and there were no clusters. The parallel control, however, was equally elevated and there was only one cluster of two. Run No. 2, however, gave lower frequencies for both treated and control. This is another example of the importance of parallel controls and repeat runs.

Table 2: Recessive Lethal Data

										Controls
Run	No. P_1 Males (1)	No. F_1 tests per stage [1/N] (2)				Total F_1 Tests	Total Lethals			% ± se
		1	2	3	4		S (3)	C1 (4)	T	
1	129					12062	14	0	14	0.116 ± 0.031
2	56					5689	5	1(2)	7	0.123 ± 0.052
3	27					1543	3	1(2)	5	0.324 ± 0.171
4	10					1386	2	0	2	0.144 ± 0.102
5	18					2732	2	2(2)	6	0.220 ± 0.115
6	24					1997	3	0	3	0.150 ± 0.087
7	30					3721	6	0	6	0.161 ± 0.066
8	27					3495	5	0	5	0.143 ± 0.064
9	25					3299	2	1(3)	5	0.152 ± 0.093
10	29					2837	4	0	4	0.141 ± 0.065
11	10					384	0	0	0	0
12	28					3216	5	1(3)1(6) 1(7)	21	0.653 ± 0.182
Total	413					42361	51	4(2)2(3) 1(6)1(7)	78	0.184 ± 0.031

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(1) No of treated or control males which produced progeny

(2) No. of F_1 females which produced progeny (lethal bearing/normal)

(3) Single lethal in progeny of one male

(4) Cluster of lethals in progeny of one male (no. of clusters/size of cluster)

Table 3: Recessive Lethal Data

Cumulative to July 18, 1975

Compound	No. PP_1 Males (1)	No. F_1 tests per stage [1/N] (2) 1	2	3	4	Total F_1 Tests	Total Lethals S (3)	Cl (4)	T	% ± se
Thimet	146	23/15450	-	-	-	15473	23	0	23	0.149 ± 0.031
E. parathion	128	16/11914	-	-	-	11930	14	1(2)	16	0.134 ± 0.035
Bromacil	117	18/6198	0/641	0/270	2/628	7757	20	0	20	0.258 ± 0.058
Captan	46	4/2075	8/1594	2/684	4/811	5182	12	1(5)	17	0.328 ± 0.117
Malathion	49	14/2593	12/1784	4/954	1/1271	6633	8	1(2)1(21)	31	0.467 ± 0.320
Guthion	44	0/2762	1/1476	1/851	5/1017	6113	7	0	7	0.115 ± 0.043
PCNB	38	2/1477	2/1089	2/518	2/854	3946	6	1(2)	8	0.203 ± 0.080
Dursban	54	5/2870	4/1906	9/1405	3/1451	7653	7	2(2)1(10)	21	0.274 ± 0.140
Trifluralin	52	5/2827	9/1531	1/1311	3/1422	7109	6	2(2)1(3) 1(5)	18	0.253 ± 0.097
Fenthion	55	8/2925	5/1950	1/1376	0/1297	7551	10	2(2)	14	0.185 ± 0.056
Azodrin	54	7/3121	2/2119	3/1585	2/1461	8276	14	0	14	0.167 ± 0.045
Folpet	30	1/1876	1/587	0/443	1/438	3347	3	0	3	0.090 ± 0.052
MSMA	29	2/2224	0/409	0/57	0/728	3420	2	0	2	0.058 ± 0.041
Dinoseb	30	4/2007	1/709	0/150	2/786	3659	3	2(2)	7	0.191 ± 0.091
Cacodylic Acid	52	2/2032	5/1978	0/1394	1/1607	7019	6	1(2)	8	0.114 ± 0.045
DSMA	52	4/2240	1/1537	2/1666	1/1378	6829	8	0	8	0.117 ± 0.041
Simazine	107	16/3922	5/999	4/1866	0/1660	8494	7	4(2) 1(3) 1(7)	25	0.294 ± 0.106
Dicamba	66	9/3034	5/1002	2/1409	1/2204	7666	5	3(2)2(3)	17	0.222 ± 0.077
Σ Controls	413	49/30309	9/3790	7/3436	13/4746	42361	51	4(2)2(3) 1(6)1(7)	78	0.184 ± 0.031

(1) No of treated or control males which produced progeny
(2) No. of F_1 females which produced progeny (lethal bearing/normal)
(3) Single lethal in progeny of one male
(4) Cluster of lethals in progeny of one male (no. of clusters/size of cluster)

Table 4: Recessive Lethal Data

Malathion

Compound	No. P_1 Males (1)	No. F_1 tests per stage [1/N] (2)				Total F_1 Tests	Total Lethals			% ± se
		1	2	3	4		S (3)	Cl (4)	T	
#1	16	12/1040	11/866	4/399	0/471	2803	4	1(2)1(21)	27	0.963 ± 0.749
#2	33	2/1553	1/918	0/555	1/800	3830	4	0	4	0.104 ± 0.052
Σ	49	14/2593	12/1784	4/954	1/1271	6633	8	1(2)1(21)	31	0.467 ± 0.320
Control #1	10	0/783	0/104	1/318	1/179	1384	2	0	2	0.144 ± 0.102
Control #2	29	2/1272	2/661	0/290	0/610	2837	4	0	4	0.141 ± 0.070
Σ	39	2/2055	2/765	1/608	1/789	4221	6	0	6	0.142 ± 0.058

(1) No. of treated or control males which produced progeny
(2) No. of F_1 females which produced progeny (lethal bearing/normal)
(3) Single lethal in progeny of one male
(4) Cluster of lethals in progeny of one male (no. of clusters/size of cluster)

Table 5: Recessive Lethal Data

Bromacil

Compound	No. P_1 Males (1)	No. F_1 tests per stage [1/N] (2) 1	2	3	4	Total F_1 Tests	Total Lethals S (3)	Cl (4)	T	% ± se
#1	89	17/5551	-	-	-	5568	17	0	17	0.305 ± 0.074
#2	28	1/647	0/641	0/270	2/628	2189	3	0	3	0.137 ± 0.079
Σ	117	18/6198	0/641	0/270	2/628	7757	20	0	20	0.258 ± 0.058
Control #1	27	5/1538	-	-	-	1543	3	1(2)	5	0.324 ± 0.171
Control #2	24	0/711	2/584	0/273	1/426	1997	3	0	3	0.150 ± 0.087
Σ	51	5/2249	2/584	0/273	1/426	3540	6	1(2)	8	0.226 ± 0.089

(1) No. of treated or control males which produced progeny
(2) No. of F_1 females which produced progeny (lethal bearing/normal)
(3) Single lethal in progeny of one male
(4) Cluster of lethals in progeny of one male (no. of clusters/size of cluster)

Table 6 shows the results of Run No. 12 for simazine, dicamba, and the parallel control. Although both compounds gave a somewhat elevated lethal frequency, this is meaningless, since the control rate was also unusually high. There were several clusters in all three groups. The conclusion is that some factor other than the pesticide treatments entered into this run. We are very concerned about this result and we are investigating possible causes. The presence of a mutagen in the environment will almost certainly be ruled out and the cause will probably be found to be genetic events in the stocks. The solution to this problem could be of considerable interest genetically and also of practical importance in connection with the avoidance of "false positives."

The overall lethal frequency for captan is not significantly high, but there are some aspects of the total picture which seem suspicious. Table 7 shows the detailed results. In both runs, the compound gave a lethal frequency of about twice the control frequency. In addition, as shown in Table 8, in both runs the other compounds gave frequencies similar to the controls while captan gave a higher frequency. (Malathion, as pointed out above, gave a frequency of 0.15 percent if the cluster is counted as one event.) We feel that captan should be investigated further.

The "chromosome mutations" (breakage, loss, rearrangement, and non-disjunction) will be discussed here only briefly, since the analysis of the data is quite involved and in any case our conclusion at this time is that none of the compounds has given a definite positive mutagenic result. As in the case of recessive lethals, each run tests two or more compounds and there is a parallel control.

Table 9 shows how the data are recorded for each run of each compound tested. The F_1 females and males are counted and exceptional flies are observed by phenotype. Clustering is noted and this can involve flies of different phenotypes and sex as well as the same phenotype in one sex, i.e., a single event can give rise to two phenotypic types.

Table 6: Recessive Lethal Data

Run #12

	No. P_1 Males (1)	No. F_1 tests per stage [1/N] (2)				Total F_1 Tests	Total Lethals			% ± se
		1	2	3	4		S (3)	Cl (4)	T	
Simazine	58					6458	7	4(2)1(3)	18	0.279
Dicamba	59					7410	5	3(2)2(3)	17	0.229
Control	28					3216	5	1(3)1(6) 1(7)	21	0.653

---

(1) No. of treated or control males which produced progeny

(2) No. of F_1 females which produced progeny (lethal bearing/normal)

(3) Single lethal in progeny of one male

(4) Cluster of lethals in progeny of one male (no. of clusters/size of cluster)

Table 7: Recessive Lethal Data

Captan

Compound	No. P_1 Males (1)	No. F_1 tests per stage [1/N] (2) 1	2	3	4	Total F_1 Tests	Total Lethals S (3)	Cl (4)	T	% ± se
#1	17	0/1024	5/971	1/388	3/395	2787	4	1(5)	9	0.323 ± 0.193
#2	29	4/1051	3/623	0/297	1/416	2395	8	0	8	0.334 ± 0.118
	46	4/2075	8/1594	2/684	4/811	5182	12	1(5)	17	0.328 ± 0.117
Control #1	10	0/783	0/104	1/318	1/179	1384	2	0	2	0.144 ± 0.102
Control #2	29	2/1272	2/661	0/290	0/610	2837	4	0	4	0.141 ± 0.070
	39	2/2055	2/765	1/608	1/789	4221	6	0	6	0.142 ± 0.058

(1) No. of treated or control males which produced progeny
(2) No. of F_1 females which produced progeny (lethal bearing/normal)
(3) Single lethal in progeny of one male
(4) Cluster of lethals in progeny of one male (no. of clusters/size of cluster)

Table 8: Recessive Lethal Data

							Two Runs			
	No. P_1 Males (1)	No. F_1 tests per stage [1/N] (2)				Total F_1 Tests	Total Lethals			% ± se
		1	2	3	4		S (3)	Cl (4)	T	
Captan	17					2787	4	1(5)	9	0.323 ± 0.193
Malathion	16					2803	4	1(2)1(21)	27	0.963 ± 0.749
Guthion	15					2697	1	1(2)	3	0.111 ± 0.083
PCNB	11					1793	2	0	2	0.112 ± 0.079
C	10					1386	2	0	2	0.144 ± 0.102
Captan	29					2395	8	0	8	0.334 ± 0.118
Malathion	33					3830	4	0	4	0.104 ± 0.052
Guthion	29					3416	4	0	4	0.117 ± 0.058
C	29					2837	4	0	4	0.141 ± 0.070

---

(1) No. of treated or control males which produced progeny

(2) No. of F_1 females which produced progeny (lethal bearing/normal)

(3) Single lethal in progeny of one male

(4) Cluster of lethals in progeny of one male (no. of clusters/size of cluster)

Table 9: Chromosome Rearrangement, Loss and Non-Disjunction

Compound Guthion (run #1) **Date** Feb. 1975

Number Treated Males 35

	Brood				
	1	2	3	4	Totals
No. F_1 Females	866	584	585	461	2496
Female Exceptions		v -♂1 y v -♂30	y v -♂9 mosaic - ♂26	y v -♂14	5
No. F_1 Males	855	550	541	475	2421
Male Exceptions	cho B^S- ♂14 y cho B^S ♂17	y cho B^S- ♂1			3
Total Progeny	1723	1137	1128	937	4925
Total Number Exceptions	2	3	2	1	8
Total Frequency Exceptions					(4 singles cl [illegible].) - ♂1 cl (2) - ♂14 16.24 x 10^{-4}

Table 10 shows the total data to date. The variation in frequency of exceptions is considerable. Only one compound, however, gave a frequency lower than the overall control. This was ethyl parathion. The rest gave frequencies higher than the control frequency although many are only slightly higher. Most of those giving the higher frequencies had clusters. Notable exceptions are methomyl (12.83×10^{-4}) and MSMA (20.29×10^{-4}). In the case of MSMA, the parallel control frequency was 5.55×10^{-4}. The sample size is still quite small, however, and is for a single run. We therefore reserve our conclusion. The sample for methomyl is that considered adequate for this test, so the result on the surface is somewhat suspicious. A look at the individual data, however, shows that in both runs the compound and control gave equivalent rates. The two control values in question are numbers 13 and 14 in Table 11. This table shows the various control runs with the chromosome mutation tests. It can be seen that the variation between runs is quite great here too, although none of the groups gave a frequency as high as some of the pesticides. There is less tendency to clustering also, but then the sample sizes are generally smaller. Thus, while there is some suggestion of a positive effect for some of the compounds, none can be proved at this point.

I would have to say, then, in summary, that under the conditions of these experiments, none of the 20 pesticides were mutagenic in either the recessive lethal test or the chromosomal mutation test. I cannot say that all of these compounds are incapable of inducing mutations in Drosophila. Perhaps if applied at higher concentration or by some other route or to some other cell type, mutagenicity could be demonstrated. Under the present conditions, however, none were proved positive.

As a scientist, I would of course like to try some of the above-mentioned variations. It is difficult to keep to a predetermined, standardized, limited screening procedure, but we must in order to be able to screen the desired number of compounds. However, there is no reason that we cannot change our standardized procedure at the appropriate time if we have found a better way to screen, a way that would increase sensitivity or facilitate the work or be more discriminating.

Table 10: Chromosome Rearrangement, Loss and Non-Disjunction

Data Cumulative to July 20, 1975

Compound	No. P_1 Males	No. F_1 Progeny	No. Exceptions	Freq. Exc. x 10^{-4}
Thimet	378	34547	19, cl (13) = 32	9.26
Ethyl Parathion	235	20721	11	5.31
Methyl Parathion	-	-	-	-
Captan	75	12676	10	7.89
Folpet	29	4655	5, cl (2) = 7	15.03
PCNB	26	4270	5	11.71
Malathion	68	11701	9, 2 cl (2), cl (3) = 16	13.67
Bromacil	118	7125	6	8.42
Guthion	65	9792	9, 4 cl (2) = 17	17.36
Azodrin	76	15030	11, 2 cl (2), cl (3), cl (4) = 22	14.64
Fenthion	84	13155	12, cl (2) = 14	10.64
Dursban	75	12385	11, cl (2) = 13	10.50
Dinoseb	23	3406	1, cl (2) = 3	8.81
Trifluralin	68	12013	9, cl (2) = 11	9.16
MSMA	30	3942	8	20.29
DSMA	50	8609	8	9.29
Cacodylic Acid	43	5937	5, cl (4) = 9	15.16
Dicamba	118	14396	10, cl (2) = 12	8.34
Simazine	151	15861	11, cl (4) = 15	9.46
Methomyl	111	20266	26	12.83
Monuron	106	15166	15	9.90
Total Treated	1929	245653	260	10.58
Total Controls	617	69767	54	7.74

Table 11: Chromosome Rearrangement, Loss and Non-Disjunction

Controls

Date Cumulative to 7-20-75

Group	No. P_1 Males	No. F_1 Progeny	No. Exceptions	Freq. Exc. $\times 10^{-4}$
1	167	12581	2	1.59
2	100	10387	8	7.70
3	25	1245	1	8.03
4	9	1485	1	6.73
5	29	3232	4	12.38
6	15	3604	2	5.55
7	20	3527	2	5.67
8	41	4365	5	11.45
9	38	6906	3	4.34
10	30	6005	3, cl (2), cl (4) = 9	14.99
11	32	3217	cl (2)	6.22
12	30	1753	2	11.41
13	35	5846	7	11.97
14	23	2208	3	13.59
15	23	3406	1, cl (2) = 3	8.81
Σ	617	69767	54	7.74

We do try to carry on some method development experiments, and I think that now that the lab is established and getting past the "growing pains" stage, we will be able to do more experimenting. There are several variations in exposure procedure that I want to look at, using known chemical mutagens and doing some comparative experiments. There are some genetic schemes that look good on the drawing board, but they must be tried in practice. Since we have just received nine more pesticide compounds, there is plenty of work to be done.

Reference

1. Vogel, E. and Sobels, F. H. "The Function of Drosophila in Genetic Toxicology Testing." (To appear in Chemical Mutagenesis, Ed., A. Hollaender, Plenum Press.)

DR. FISHBEIN: I realize insolubility is a problem with pesticides. I'm a bit apprehensive of the use of DMSO.

DR. VALENCIA: So are we. We are using it in desperation. We didn't know what to do with these things. We can't get them in solution.

DR. FISHBEIN: You couldn't vouchsafe the stability of some of these materials in DMSO, that in point of fact you are testing captan, per se, and not some degradation products after it's been sitting in DMSO. Have you tried any other solvents?

DR. VALENCIA: No, we haven't. This is one of the things that I would like to try.

COMMENT: I just want to remark that of the substances you tested, we have tested only two in the Salmonella work we've done, because we weren't working on pesticides.

Those are PCNB and captan. Captan is clearly positive, and PCNB is negative. And so the suspicion that you have about captan, I think, will be well worth additional efforts so that you can build up your statistics.

DR. VALENCIA: One of the bases for my suspicion of captan is simply that it has come up positive in a lot of different systems. Some negative results in Drosophila have been published, but I, for one reason or another, have reservations about those negatives. So it really does need another more intensive look.

COMMENT: I would prefer to think about the suspicion as being related only to your data, and certainly your data indicate that of all the things you've tested, that's the one that's approaching a significant effect. So I really think you ought to just plug away at that one. It's obviously very important economically.

DR. VALENCIA: You just reminded me of one thing that I forgot to point out. You may have noticed that the standard errors were very large in some of the cases. This is because in the cases in which we have found clustering, we apply a formula which was developed by Dr. H. J. Muller to take into account that there are mutations which probably are of common origin. This simply wipes out the significance whenever you happen to hit a big cluster.

IN VITRO AND IN VIVO STUDIES OF SELECTED PESTICIDES TO EVALUATE THEIR POTENTIAL AS CHEMICAL MUTAGENS

Dr. Gordon W. Newell

The Federal Insecticide, Fungicide, and Rodenticide Act designates EPA as the Governmental body responsible for the safety of all pesticides used in the United States. More recently, the Federal Environmental Pesticide Control Act (PL 92-516) identified EPA's regulatory responsibilities in the area of pesticides to include intra- as well as interstate commerce.

To be Federally registered, a pesticide must be determined to be not hazardous to health or to the environment when used according to its labeling restrictions. Thus, relative to new law, as well as to specific directives included in Public Law 93-135, 1973, EPA is now reviewing the use of alternate chemicals for pest control, including older registered pesticides.

In the EPA pesticide review process, emphasis is placed on development of scientific criteria for evaluating the safety of compounds, substituting those pesticides found to be hazardous. In addition to reviewing and evaluating the pesticide literature, as well as maintaining liaison with industry and academia, the program includes laboratory studies to obtain additional data to support the literature review effort. One of these laboratory programs is directed toward gathering mutagenesis data on a selected number of compounds. Stanford Research Institute is applying its best efforts in conducting this study.

Development of methods to evaluate the mutagenic hazard of chemical compounds has advanced markedly in the last few years. From undefined empirical tests of a short time ago, procedures are now available which can detect chromosome breaks and genetic

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changes caused by chemical stress. Mutant strains of microorganisms in cell culture and mammalian fibroblast cells in tissue culture are effective in vitro systems for reliable evaluation of presumptive mutants, while the mammalian dominant lethal test, using mice or rats, is a recognized test for the assessment of chromosome damage to germinal cells.

Today there are many pesticide chemicals in commercial use which have not been adequately investigated for their mutagenic hazard. With the increasing concern by the public about possible stress of chemicals to our environment, there is need for such studies of the widely used pesticides. This project is utilizing test methods which are appropriate for these evaluations and which are in use by the scientific community.

Objective and Scope

The objective of this program is to investigate the mutagenic potential of selected pesticides, using in vitro and in vivo test procedures.

To investigate the mutagenic potential of selected pesticides, the following three test systems are used:

- Microbial assays with and without metabolic activation
- Human fibroblast cell culture assays for DNA repair with and without metabolic activation
- Dominant lethal tests in mice

In Vitro Mutagenesis Studies

Microbial Indicators of Mutagenesis

Numerous systems are available for studying chemical mutagenesis, and they have been described in detail. Each system has specific advantages and disadvantages, but certainly no one system can give a true estimate of the total effects on man -- our primary concern. Considerations such as uptake, metabolism, detoxification, activation, type of exposure, repair mechanisms, and dosages are difficult to evaluate by any one test system. However, from the standpoint of time and economics, it is unrealistic to use a wide range of test systems to examine all chemicals to which man may be exposed.

Microbial systems, including viruses, prokaryotes, and eukaryotes, have been used for chemical mutagenesis studies. In addition to straight microbial systems, combination systems allowing for metabolic activation have been developed, such as the host-mediated assay and the microsomal activation systems.

There are many advantages to using microorganisms since they can be very sophisticated and yet extremely sensitive, simple, and economical. On the other hand, they are non-mammalian and, therefore, their validity with regard to man has been questioned. Microbial systems lack the metabolic activation potential of man, and there are questions related to the repair potential between microorganisms and man. However, many of these questions can be circumvented by using mammalian metabolic activation systems and by eliminating repair mechanisms. Furthermore, since the site of mutations is DNA and since there is a homogeneity or uniformity with regard to the chemical and physical composition of all living systems, an adverse effect in one system may represent a potential threat to other living systems.

Microsomal Activation Systems

The conversion of a compound to an active metabolite is a requirement in the detection of certain mutagens or carcinogens. Hepatic microsomal enzymes are very active metabolically and have been shown to activate various compounds.

Malling and Chu have used liver microsomes as an activation system with mammalian cell cultures or microorganisms as the indicator organism. Chu has reported that the combined treatment of dimethylnitrosamine (DMNA, a potent carcinogen in rodents thought to be metabolized via oxidative dealkylation before becoming tumorigenic) with liver microsomes produces a significant increase in the frequency of azaguanine - resistant mutant hamster cells in tissue culture, over treatment with DMNA alone. Similarly, Malling showed increased mutation frequencies for the reversion of histidine auxotrophs of *Salmonella typhimurium* to prototrophy when cells, liver microsomes, and dimethylnitrosamine were incubated together.

Slater *et al.* also have shown that a liver microsomal fraction potentiates diethylnitrosamine and dimethylnitrosamine activity in systems utilizing bacteria deficient in DNA polymerase; such systems are more sensitive to agents that alter cellular DNA than is the parent strain from which they were derived.

Ames has incorporated a rat microsomal activation system with his tester strains of histidine auxotrophs of *Salmonella typhimurium* in studying the mutagenic effect of chemicals. In this procedure the microsomes are included in the top agar along with the bacterial test strain and the chemical being tested. Using the Ames method, we have activated 4-aminobiphenyl, 2-amino and 2-acetylaminofluorene, aminoanthracene, aminopyrene, methyl and dimethyl benzanthracene and benzpyrene to mutagenic metabolites.

As part of this contract, we have determined the mutagenicity of 20 proposed substitute chemicals toward microorganisms (Table 1). Three types of mutagenicity have been measured:

1. Qualitative changes in the information encoded in the DNA (Reverse mutation in *Salmonella typhimurium* and *E. coli* WP2).
2. Chromosomal changes in *Saccharimyces cerevisiae*.
3. The toxicity of chemicals as a function of the absence of various DNA repair systems. These studies were conducted in *B. subtilis* and *E. coli*.

Table 1: EPA Pesticides - Contract No. 68-01-2458,
SRI Project 3493

Folpet
Captan
Methyl parathion
Ethyl parathion
Malathion
PCNB
Bromacil
Azodrin
Guthion
Thimet

Cacodylic acid
MSMA
DSMA
Dursban
Trifluralin
Fenthion
Dinoseb
Methomyl
Simazine
Monuron

Table 2: Pesticides Showing a Positive Mutagenic Response in One or More In Vitro Systems

Pesticide	S. typhimurium	S. cerevisiae	E. coli	B. subtilis
Captan	+	+	+	-
Folpet	+	+	+	-
Bromacil	+	+	+	-
Azodrin	-	+	-	-
Guthion	-	+	-	-
Cacodylic acid	-	+	-	-
Dursban	-	-	+	-
Dinoseb	-	-	+	-

The following are characteristics of the first type of assay system, whether it is conducted in Salmonella or Escherichia. The bacterial strain contains a mutation which results in a requirement of an amino acid for growth. Thus, in the absence of the exogenous addition of the amino acid, the microorganism cannot grow unless the mutation is specifically reverted. The advantage of this assay system is that only mutants grow. Thus, the frequency of mutation can be measured by counting those bacteria which can give rise to colonies.

Two types of mutation are known to be specifically reverted by chemical mutagens. The first type is called base-pair substitution. A base-pair substitution occurs when a base, e.g., adenine, is replaced by one of the other three bases: thymine, guanine, or cytosine. The second type of mutation is a frame-shift mutation. The information encoded in the DNA is read (converted into protein) by starting at a fixed point and reading three bases. Thus, we speak of a reading frame of three bases or a triplet, which specifies an amino acid. If one (or two) bases are lost or gained, the reading frame from that point on will be out of phase. Thus, each subsequent triplet will specify a wrong amino acid. Polycyclic hydrocarbons can become inserted in the DNA and cause frame-shift mutations. Chemicals which tend to cause the loss of one base will revert mutations resulting from the addition of one base or the addition of two bases. E. coli WP2 and S. typhimurium strains TA1535 and TA100 are base-pair substitution mutations. They are reverted by many alkylating chemicals. S. typhimurium strains TA1536, TA1537, and TA 1538 are frame-shift mutants and are reverted by polycyclic aromatic hydrocarbons.

Strain TA100 is derived from TA1535. It contains independently replicating DNA elements (plasmids) which, by a mechanism that is not currently understood, increase its sensitivity to mutation by some chemicals.

The second type of mutagenic activity we have measured is mitotic recombination in S. cerevisiae. Mitotic recombination defines an event in which there is an exchange of chromosomal material between homologous chromosomes. It implies that these have been broken, simultaneously or sequentially, in each of the chromosomes. Therefore, chemicals which increase mitotic recombination may be identified as chemicals which potentially increase chromosome breakage. Chemicals which cause aberration in the mechanism involved in the distribution of chromosomes to daughter cells might also be scored (incorrectly) as mitotic recombinants. In general, chemicals which tend to increase mutation also increase mitotic recombination.

The types of mutagenesis detected by the above mutagenic assay systems are relatively specific, e.g., base-pair substitution or a frame-shift mutation within a specific gene of the histidine operon in Salmonella, chromosomal exchange between the centromere and a specific gene.

Methods

Bacterial Mutation

The histidine requiring Salmonella typhimurium strains TA100, TA1535, TA1536, TA1537, and TA1538 are used in all in vitro assays. Strains TA100 and TA1535 are particularly sensitive to chemicals that cause base-substitution mutations; and strains TA1536, TA1537, and TA1538 are sensitive to chemicals that cause frame-shift mutations. All tests are performed at five dose levels (1, 5, 10, 50, 100, 500 and 1,000 μg per plate) and in the presence and absence of a metabolic activation system. In the absence of metabolic activation, approximately 5×10^8 bacteria are added to 2 ml of top agar (0.6 percent Difco agar, 0.6 percent NaCl, 0.050 mM of L-histidine, 0.05 mM of biotin). The test chemical is added to the top agar, mixed, and then poured onto the surface of a minimal agar plate, which is composed of Vogel-Bonner E medium, 1.5 percent agar and 2 percent glucose.

The metabolic activation system consists of 0.15 ml of the 9000 x g supernate of homogenized rat liver, $MgCl_2$, KCl, glucose-6-phosphate, TPN and sodium phosphate buffer (pH 7.4); 0.5 ml of this mixture is added directly to the top agar immediately before it is poured over the minimal agar plate. The plates are incubated at 37°C for 40 hours. The number of his^+ revertant colonies on each plate is then counted.

To determine if the test chemical is toxic to the bacteria, the following modification of the above technique is used. Approximately 300 bacteria are added to the top agar and the concentration of L-histidine in the top agar is increased to 1 mM. After overnight incubation, the number of colonies in each plate is determined. A significant reduction in the number of colonies formed in the presence of the test chemical when compared to the controls indicates that the test chemical, or a metabolite of it, is toxic.

Mitotic Recombination in Yeast

The ade^- heterozygote *Saccharomyces cerevisiae* D3 is used in all *in vitro* assays. The D3 strain is incubated for 4 hours at 30°C in phosphate buffer with the test chemical at a concentration which provides a 50 percent kill, or at a concentration of 5 percent, whichever is lower. Dilutions of 1:5 and 1:25 of the highest test concentration are also tested. When present, the metabolic activation system is the same as that used in the bacterial assays.

After treatment, the sample is diluted serially in sterile saline and plated on tryptone-yeast agar plates. Plates of a 10^{-3} dilution are incubated for 2 days at 30°C followed by 2 days at 4°C to enhance the development of red pigment, indicative of ade^- homozygosity. Colonies are examined for the presence of red colonies or red sectors by scanning the plates with a dissecting microscope at 10x magnification. Plates of a 10^{-5} dilution are incubated for 2 days at 30°C to determine the total number of colony forming units.

When a metabolic activation system is present, the yeast suspension is incubated for 4 hours at 37°C with the test chemical. The metabolic activation system is the same as that used for *Salmonella*.

All experiments are performed in duplicate. Both positive and negative (solvent) controls are included in each experimental run. Positive reference compounds include 4-0-tolylazo-o-toluidine and MNNG.

Additional test systems are now being evaluated; also attempts are underway to improve the sensitivity of the yeast assay system.

B. subtilis Assay (Recombination-Deficient Strain)

Kada has reported on several experiments in which mutagenic chemicals have been tested by the "Rec-assay" (1,2,3). This assay is based on the differential sensitivity of bacteria to agents that interact with DNA. The origin of the differential sensitivity is the introduction of mutations in one of the pathways for repairing damaged DNA. One such pathway is the repair of DNA by recombination. This assay system has been used by Kada for mutagenic screening of pesticides (2). However, Dr. Kada reported at the joint United States-Japan Conference on Chemical Carcinogens (December 9-11, 1974) that he had not incorporated a metabolic activation system into his procedure.

We are testing five chemicals known to require metabolic activation to develop this system. Each chemical is tested using the following three procedures to determine which gives the most sensitive response:

1. The metabolic activation system is incorporated into the agar (as in the Salmonella assays) with the bacteria, and the test chemical is placed on a disc in the center of the plate.
2. The metabolic activation system is incorporated into the agar and the test chemical is placed in a well in the agar.
3. The metabolic activation system and the chemical are placed in a well in the agar.

The five chemicals we are testing are 1) dimethylnitrosamine; 2) β-naphthylamine (β-naphthyl-hydroxylamine will be used as a positive control); 3) 4-aminobiphenyl; 4) N-2-fluorenylacetamide; and 5) 3'-methyl-4-dimethylaminoazobenzene. Dimethylnitrosamine causes base-pair substitution mutations, β-naphthylamine causes base-pair substitutions and frame-shift mutations; and the last three chemicals cause only frame-shift mutations. These experiments also are conducted in the absence of metabolic activation.

E. coli Polymerase Assay(4)

The principle of this technique is the same as that decribed for *B. subtilis*, except that the repair system that has been inactivated is the DNA polymerase gene *polA* rather than the recombination genes.

E. coli WP2 Reverse Mutation Assay

The principle of this technique is the same as that for the *Salmonella* assays. *E. coli* WP2 is a tryptophan auxotroph (*try*$^-$) that can be reverted to tryptophan prototrophy by mutagens that cause base-pair substitutions (5, 6). The protocols for these experiments are identical to those described for *S. typhimurium*, except that tryptophan is substituted for histidine in the top agar.

Development of a More Sensitive Yeast Assay System

A number of carcinogens, e.g., auramine o, have been detected as mutagens in assays with *S. cerevisiae* D3 that have not been detected in bacterial assays. Furthermore, the assay for mitotic recombination measures chromosome damage and subsequent mitotic crossing-over. However, the primary disadvantage of this system appears to be that the yeast cell wall is impermeable. We have observed that many carcinogens require metabolism to reach active form (procarcinogens such as 2-anthramine and 4-aminobiphenyl) but do not increase mitotic recombination.

We are approaching the permeability problem in two ways:

1. Testing the feasibility of using glusulase, an enzyme from the snail gut, in order to increase the permeability of the yeast cell wall to compounds under test.
2. Cell wall-defective yeast mutants. We have received three strains of yeast from Dr. Clint Ballou of the University of California at Berkeley. Two of the strains are defective in mannose polysaccharide biosynthesis. We are comparing these strains

with their parent strain for sensitivity to a variety of polycyclic compounds to determine whether their permeability is increased. If the mutants are more sensitive, we will obtain or genetically construct adenine heterozygotes to determine whether the sensitivity to mitotic recombination also is increased. The compounds being tested in this phase are 4-aminobiphenyl, 4-nitrobiphenyl, and 2-anthramine.

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UNSCHEDULED DNA SYNTHESIS TESTING OF SUBSTITUTE PESTICIDES

Dr. Ann D. Mitchell*

Unscheduled DNA synthesis (UDS), the incorporation of nucleotides to repair DNA damage, has been observed in all stages of the cell cycle and occurs in a wide variety of mammalian cell types. This repair (or misrepair) of DNA damage may be considered to be an important type of early genetic event leading to cytogenetic aberrations, to mutagenesis, and/or to carcinogenesis.

Under contract to the National Cancer Institute, we recently developed a new UDS assay system for prescreening chemical carcinogens. Currently, this system is being validated through use of a series of chemicals that have shown in animal bioassay tests to be ultimate carcinogens, procarcinogens, and noncarcinogens. The efficiency and economy of the new assay system are compatible with the requirements for short-term prescreening, since the results of each test can be obtained in 2 to 3 days. Moreover, we have found that the sensitivity and reliability of this method are greater than that observed in the measurement of DNA repair synthesis by other prescreening approaches. To date, for approximately 50 chemicals, a greater than 95 percent correlation has been observed between UDS assay results and the bioassay results. Therefore, we have used the UDS assay system to test 20 substitute pesticides for the Environmental Protection Agency.

For the UDS assays, we use diploid human fibroblasts (WI-38 cells) contact-inhibited in the G_1 phase of the mitotic cycle by depletion of serum in the medium. Since a low percentage of cells still may escape synchrony, 10^{-2} M hydroxyurea is added to inhibit scheduled (S-phase) DNA synthesis that otherwise would obscure measurement of the "unscheduled" repair of DNA damage. We have used autoradiography to check the combination of these two approaches and have found that no greater than 0.2 percent of the nuclei are in S-phase at the time of the assays.

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In preparation for the assays, fibroblasts are grown for 2 weeks in small (T-25) tissue culture flasks. Compounds are then tested in the presence and in the absence of a metabolic activation system with replicate samples for each concentration of a series of log dilutions. Appropriate positive and negative controls are tested with each compound. The positive control, without metabolic activation, is 4-nitroquinoline-N-oxide; dimethylnitrosamine is used as a positive control with metabolic activation; and solvent only is the negative control. In the absence of metabolic activation, we expose the cells to the compounds and to tritiated thymidine (^{3}H-TdR) for incorporation in the repair of DNA damage for 3 hours. However, an exposure of only one hour is used when a metabolic activation system is added, because we have found that the liver homogenate (used as a source of metabolic activation enzymes) also may contain substances toxic to tissue culture cells. Following the compound exposure, the samples are rinsed thoroughly and incubated with media containing unlabeled thymidine to remove excess ^{3}H-TdR. The cells then are removed from the flasks, and DNA is extracted using perchloric acid. An aliquot of the DNA solution is used to measure incorporation of ^{3}H-TdR by scintillation counting. A second aliquot is used to determine the DNA content by measuring absorbence at 600 mμ following reaction with diphenylamine reagent. The results can be expressed per unit of DNA and are compared with the background rate of DNA repair. That is, we calculate the mean, standard deviation, and standard error for each concentration and then determine the fold increase for that concentration relative to the negative control.*

We have completed preliminary UDS testing, with and without metabolic activation, of the 20 substitute pesticides, and these results are presented in Table 1.+ Two unique observations are that:

1. Positive responses were observed for six compounds. Metabolic activation was required to obtain positive responses for five compounds; only one substitute pesticide was positive in the presence and absence of metabolic activation.

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* DPM/μg DNA of treated cells divided by DPM/μg of negative control.

+ This testing was completed after the paper was presented and thus has been added.

Table 1: Preliminary UDS Results of Testing 20 Substitute Pesticides

-MA, Without Metabolic Activation
+MA, With Metabolic Activation

Compound	UDS Results	
	-MA	+MA
Azodrin	+	+
Bromacil	-	+
Cacodylic acid	-	-
Captan	-	+
Dinoseb	-	-
DSMA	-	-
Dursban	-	-
Ethyl parathion	-	-
Fenthion	-	-
Folpet	-	+
Guthion	-	+
Malathion	-	-
Methomyl	-	-
Methyl parathion	-	-
Monuron	-	+
MSMA	-	-
PCNB	-	-
Simazine	-	-
Thimet (phorate)	-	-
Trifluralin	-	-

2. No strong positive responses such as the 20- to 40-fold increases we commonly observed for positive control compounds were observed for the substitute pesticides. Rather, all positive responses were approximately two-fold increases. This low level of DNA repair might not have been detected with other, less sensitive approaches.

MAMMALIAN SCREENS

Dr. Gordon W. Newell

This part of our presentation describes the work we have done with mammalian systems and which we are extending. Initially, let me review the dominant lethal procedure and indicate a few modifications we have made in the procedure over what is generally used in the literature; then I will continue with the results and explain what is coming up in this next year.

Dominant Lethal Procedure

All compounds were supplied by the Environmental Protection Agency.

Although acute toxicity information of some of the compounds was available in the literature, confirmatory tests were conducted to obtain an LD_{50} under the conditions of this laboratory. If no data were available, a range-finding dose regimen was initially conducted, followed by an accurate determination of the oral LD_{50}.

The solubility of each compound is tested with various agents such as water, propylene glycol, polyethylene glycol, corn oil, tricaprylin, carboxymethylcellulose, or methylcellulose (Methocel) to determine the most appropriate vehicle for administration.

Based upon single dose acute toxicity data, several dose levels of the test compound are administered in feed for a period of 2 weeks for the purpose of establishing doses for the 7-week exposure period. Criteria for a maximum tolerated dose include failure to gain weight, development of clinical signs, and mortality. The maximum dosage level selected may produce up to 20 percent weight decrement and mild but transient clinical signs with no mortality.

For the dominant lethal study, three dose levels are administered. The highest dose is the maximum tolerated dose, or percentage thereof, that does not inhibit breeding performance. This will be determined by caging treated males with virgin females and inspecting the females for the presence of vaginal (mating) plugs. Comparison is made with vehicle-treated males. Two approaches are used for the selection of middle and low doses:

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1. The intermediate dose is 1/10 of the selected maximum dose with the low dose 1/100 of the maximum dose, or

2. The middle and low doses are one-half and one-quarter of the highest dose, respectively.

Adult male and female ICR/SIM mice are used. Males are proven breeders, while the females are of virgin stock.

Each experimental group consists of 20 adult male mice. After being fed the compound in the diet for 7 weeks, each male is mated with two adult virgin female mice for a period of 7 days. Females are replaced weekly for 8 weeks. Purina Lab Chow and water are available to the animals at all times.

An unsupplemented group is included in each experiment. Animals in this group are treated in the same manner as are the test-compound groups.

The positive-reference control group is treated with known mutagen, triethylenemelamine (TEM), by means of a single intraperitoneal injection of 0.2 mg/kg. Breeding and implant data are obtained throughout the 8-week study.

Pregnant females are sacrificed over a 4-day period starting on the 15th day after the first day of breeding. This schedule allows for the sacrifice of females between the 11th and 17th days of gestation. A complete autopsy of each female is done to determine if an incurrent infection is present, since such a condition can induce pre-implantation loss and early fetal deaths. Infected animals are excluded from the experiment.

At the time of sacrifice, each female is scored for early fetal deaths, late fetal deaths, and living fetuses (all of which provide a total implant score).

The following parameters indicate an effect in dominant lethal studies: total implants (live fetuses plus early and late fetal deaths), total dead (early and late fetal deaths), and dead implants per total implants. All data are analyzed for statistical significance by the t-test.

Results

All 10 pesticides studied by the dominant lethal procedure in ICR mice produced negative responses. These were parathion, methyl parathion, captan, Thimet, folpet, malathion, PCNB, bromacil, Azodrin, and guthion.

New Oral Reference Mutagens

The major reference mutagen for mammalian studies is triethylenemelamine (TEM). It has been shown to be effective in producing dominant lethal effects, and also causes heritable mutagenic responses in the translocation test. Not only is it active when administered intraperitoneally, it is also effective by the oral route when administered in the drinking water. However, this compound is available only from one source, a pharmaceutical manufacturer, and the company has decided to stop production. Other compounds are effective mutagens when given by injection, but data are lacking when they are administered per os. Since a major route of pesticide hazard is by ingestion, it was decided to investigate additional compounds as candidate oral reference mutagens.

In this portion of the program we are studying the following compounds: cyclophosphamide, ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), tris (1-aziridinyl) phosphine oxide (TEPA), tris [1-(2-methyl aziridinyl)] phosphine oxide (METEPA), and trimethyl phosphate (TMP). Where necessary, we are testing LD_{50}s to determine the oral acute toxicity of each material.

Based on this information as well as other data available from the literature regarding dose levels shown to cause positive mutagenic responses when the compound is administered intraperitoneally, several dose levels are selected for a short-term dominant lethal study. For these compounds, dose levels are administered in the feed to adult male mice for 2 weeks so that a dose response useful for subsequent breeding can be established. At the end of the 2-week exposure period, the males are caged with virgin females, and the females are examined daily for the presence of vaginal (mating) plugs. At mid-term of pregnancy, the females are sacrificed and examined for total implants, as well as for early and late fetal death. These initial screenings should indicate dosage levels which do not affect breeding performance and will identify effective dosages for mutagenic activity by the oral route of administration.

Following a careful evaluation of the preliminary screening data, potentially active dose levels are selected for a more extensive dominant lethal test. In our experience with other chemicals, we have found it is necessary to study a broad range of dosages inasmuch as overt toxicity may not be apparent, but inhibition of breeding and sterility can occur.

In the more extensive dominant lethal study, the test compound is administered to adult males at several dosage levels in the diet for 8 weeks. At the end of this time, treated males are caged with virgin female mice and the females are examined daily for the presence of vaginal (mating) plugs. Evaluations for mutagenic responses are conducted as indicated above. Results obtained at this phase should indicate dosage levels that do not affect breeding performance but identify effective dosages for mutagenic activity by the oral route of administration. As such, the following conditions may exist: 1) one or more dose levels show positive oral mutagenic activity, in which case the compound is retained for validation in the translocation procedure; and 2) none of the dosage levels tested indicate mutagenic activity. No further work is done on that compound

Based on positive response results obtained in the dominant lethal studies of candidate oral mutagens, two dose levels of the compound will be used in the translocation procedure. A high dietary level is the maximum tolerated dose (MTD) and the low level is one-fourth of the MTD.

Adult male proven breeder mice, ICR/SIM strain, are fed the test compound at the selected dietary levels for 8 weeks. We also use an untreated control group as well as a group treated with the standard reference mutagen, triethylenemalamine (TEM); the TEM treatment is for 4 weeks, with the compound being added to the drinking water and fresh solutions prepared daily.

At the end of the treatment period, 25 to 30 males from each group are mated to 2 virgin females each for 1 week. These females are allowed to deliver their offspring; the parent males are discarded.

Our experience indicates that this breeding schedule should provide 500 to 600 offspring. The litters are raised to weaning age, at which time the females are discarded, and the F_1 males are raised to maturity (11 weeks of age).

At maturity, 100 F_1 males from each group are mated to 3 virgin females each for 2 weeks. We examine females daily to identify the breeding date (presence of vaginal plugs) Fourteen days after confirmed mating, the females are sacrificed and examined for total implants, as well as for early and late fetal deaths. Males bred to females producing litters which do not meet "normal criteria" are considered as suspect translocate animals. Each of those males is rebred to three additional virgin females. We evaluate the second breeding in the same manner as the first breeding.

Our criteria for classifying a male as "partially sterile," "sterile," or "nonbreeder" are:

- Partially sterile male: If all three females are pregnant, each must have nine or less live implants, with at least one having six or less live implants. If two of three females are pregnant, both must have nine or less live implants, with one having six or less live implants. If only one of three females is pregnant, this female must have six or less live implants.
- Sterile male: None of three females are pregnant -- previously identified by presence of vaginal plug.
- Nonbreeder male: None of three females are pregnant -- not previously identified by presence of vaginal plug.

Any F_1 male not fitting one of these criteria is considered normal and discarded.

For each male meeting the criteria of sterile or partially sterile after a second breeding, we conduct cytogenetic evaluations. Such evaluations include measurement of testes weight, meiotic metaphase I cell evaluation for translocations, and preparation of photomicrographs for each animal studied.

Now I will show you where we are at the present time in the evaluation of some of these candidate oral reference mutagens.

Table 1 shows the results with cyclophosphamide. We are conducting a screen and as a result you will not see control animals, but we do have a good reference data base. We know that the average implants of a control female are in a range of 10.4, plus or minus 0.2. The average dead implants per female are normally around about 0.2 to 0.4 in our experience. So, if we see an increase of implants, we know that the response is suggesting a mutagenic event.

Table 1: Preliminary Breeding Data From Male Mice Treated Orally with Cyclophosphamide

Dose (mg/kg) Single	Week	% Pregnant	Average Implants/Female	Average Dead Implants/Female
125	1	70	9.86	5.57
	2	60	9.67	0.50
250	1	20	7.50	4.50
	2	40	11.25	4.00
500	1	17	15.00	0
	2	33	8.00	1.00
Multiple				
37.5	1	38	11.33	3.67
	2	50	9.25	3.00
	3	50	5.67	1.33
75	1	60	4.17	2.67
	2	30	5.33	2.33
	3	40	14.25	7.75

These are relatively small numbers. We are looking at single dose data, trying to find a tolerated level. The multiple system here is five doses, repeated daily, at this level per kilogram per day.

It is obvious in looking at the repeat data that these dose levels are markedly reducing the litter size and causing an increase in dead implants. Again, with 75 mg/kg of cyclophosphamide, there is a marked reduction of implant frequency, where we would like to see a normal number of implants but a significant increase in dead implants. This is really too toxic a level.

Tables 2 and 3 show the same type of response with EMS and MMS. There appear to be problems of cumulative effects, and I question whether we can use these materials successfully.

Table 2: Preliminary Breeding Data From Male Mice Treated Orally With EMS

Dose (mg/kg)	Week	% Pregnant	Average Implants/Female	Average Dead Implants/Female
Single				
125	1	80	11.38	0.25
	2	100	14.70	1.00
250	1	60	10.83	0.17
	2	50	11.20	0.60
500	1	90	7.89	2.56
	2	70	11.00	0.86
Multiple				
125	1	70	10.57	4.86
	2	80	11.38	4.50
250	1	0	-	-
	2	30	9.33	2.00

Table 3: Preliminary Breeding Data From Male Mice Treated Orally With MMS

Dose (mg/kg)	Week	% Pregnant	Average Implants/ Female	Average Dead Implants/Female
Single				
125	1	60	5.33	2.50
	2	90	10.67	0.67
250	1	20	2.50	2.50
	2	90	10.22	2.89
315	1	0	--	--
	2	31	6.20	2.60
Multiple				
63	1	0	--	--
	2	20	9.50	3.00

Tables 4 and 5 show similar data for TEPA and METEPA. In Table 6 the preliminary responses with TMP appear promising and we have hopes that this compound may eventually prove to be a satisfactory new oral reference mutagen.

Table 4: Preliminary Breeding Data From Male Mice Treated Orally With TEPA

Dose (mg/kg)	Week	% Pregnant	Average Implants/Female	Average Dead Implants/Female
Single				
25	1	80	9.13	4.63
	2	80	13.13	0.88
50	1	40	6.00	4.00
	2	70	13.29	1.86
125	1	10	10.50	8.00
	2	40	8.13	2.75
Multiple				
25	1	10	3.00	3.00
	2	50	6.00	4.60
	3	40	9.00	6.00
50	1	0	-	-
	2	20	1.50	1.50
	3	10	12.00	12.00

Table 5: Preliminary Breeding Data From Male Mice Treated Orally With METEPA

Dose (mg/kg)	Week	% Pregnant	Average Implants/ Female	Average Dead Implants/Female
Single				
50	1	50	13.6	1.60
	2	80	14.5	3.00
100	1	60	7.33	5.17
	2	70	7.86	4.00
300	1	17	5.33	4.67
	2	25	5.75	4.50
Multiple				
31.25	1	80	10.5	2.50
	2	70	11.86	1.71
	3	67	9.33	1.67
62.5	1	80	10.25	4.63
	2	70	7.86	2.71
	3	30	2.00	0.33

Table 6: Preliminary Breeding Data From Male Mice Treated Orally With TMP

Dose (mg/kg)	Week	% Pregnant	Average Implants/Female	Average Dead Implants/Female
Single				
2000	1	50	10.00	0.80
	2	70	11.29	0.86
2500	1	50	6.40	3.00
	2	40	7.50	0.50
3500	1	17	7.00	6.00
	2	83	13.60	1.00
Multiple				
750	1	38	7.67	6.00
	2	63	10.60	7.60

DR. CURLEY: Since the toxicant first comes in contact with epithelial cells and is absorbed or transported by some transport mechanism and detoxified, or metabolically altered, what is the feasibility of developing, validating, and utilizing such cell culture systems which would approximate the normal cellular exposure to the toxicant in the animal, rather than using fibroblasts for mutagenic or carcinogenic _in vitro_ screens?

DR. MITCHELL: I think several laboratories are initiating work on cell transformation in epithelial cells. Results from these studies might be anticipated in one and a half or 2 years.

DR. CASCIANO: Dr. Weinstein is developing epithelial cell systems, and several laboratories are developing rat liver epithelial type cultures, and they're showing a very good correlation between ability to grow in soft agar and ability to form carcinomas in the whole animal.

QUESTION: This is a question to Dr. Newell. When you're doing these studies with high dosages, instead of repeated doses -- do these pesticide chemicals on oncogenesis really transform the cells by selecting somehow the pre-existent malignant cells, or schussing on to the oncogenesis virus that carries out this transformation?

DR. NEWELL: As I understand your question, you are asking about mutagenesis responses by pesticides in mammalian systems. With mammalian systems, we have not found a positive response yet, so I don't think one can give you a direct answer. With the microbiological systems there appears a direct effect on the DNA.

QUESTION: I'd like to address a comment and a question to Dr. Newell. We all know that there's a multitude of possible microbiological screens, both natural and mutant, and it would seem that if we go into a study with a preconceived view that a compound is a carcinogen, mutagen, teratogen, what have you, that by analogy with another compound, with another chemical, by a gut feel, or whatever criteria seem to be in vogue, we'll never put the question to rest. My question is how are several negative findings and one or two positive findings ever going to answer this question. In other words, when is a compound finally going to be cleared of being a presumptive carcinogen, mutagen, or what have you?

DR. NEWELL: I think this is a very proper question from someone concerned with the problem of registering compounds and getting them approved. As I commented earlier, one of the main reasons for these large amounts of data developed with known carcinogens and noncarcinogens has been to develop validity for the system. The data developed with all these compounds have come from investigators at universities, NIH, and NIEHS, and organizations like ourselves, all attempting to validate the systems.

These kinds of information are for reference, and are not intended to intimidate anyone. Whether or not they are directly meaningful to a pesticide you may be concerned with is secondary. The information developed for those compounds is one way to show that the procedures have some validity for what we're attempting to do.

Some people have suggested that more of our studies should be done with the compounds under code,. Some say when you know the compound your results will be what you want to get. Hopefully, that's not raising a question of integrity, but I think it's a proper question to ask.

When we look at new chemicals and new structure types, unless we have enough information about compounds active in mammalian systems (and if you are trying to compare mammalian results with the in vitro ones), unless you have a reliable base of reference, none of the information is meaningful. Today, the correlation, the mutational responses of the in vitro microbiological systems are 80 to 85 percent from studying chemicals known to cause carcinogenesis responses in animals.

You asked if there is a positive _in vitro_ response, will it ever get put to rest. If one is concerned, one can use other systems for confirmation, i.e., cytogenetic studies or the translocation test.

Actually we've done a number of translocation studies on drugs and agricultural chemicals, providing heritable-type experimental data on such products.

DR. COMMONER: I'm glad this question came up, because it's the nitty gritty, and although I will present considerable data that read on this after lunch, I do want to make a comment here.

Let's simply empirically say you have a compound that you want to put out in such a way that people will be exposed to it. You want to be sure that it's not a carcinogen. Now I define a carcinogen as something that causes cancer in people. So you want to be very sure that it doesn't cause cancer in people.

At the present time there is literally no way of doing that, short of causing cancer in people. So if you have industrial exposure data, then you can say something about it. Now obviously that's true of very few compounds.

What can you do? You can put it through an animal test. That tells you that it causes cancer in mice and rats, and it does not necessarily tell you that it causes cancer in people. However, we know that a lot of things that cause cancer in mice and rats do cause cancer in people, and it's a big warning sign.

Now the trouble with doing that is that it is fantastically expensive and slow. The last figures we got in Seattle from NCR were an average of $150,000 a compound and 3 years of work.

There is something else that can be done, and that's where all of the _in vitro_ and other tests come in. Suppose we could give you a fast test that will take 2 days and cost $500.00 a compound, in which the bacteria stand up on end and whistle Dixie if they're exposed to a carcinogen, and if it's not a carcinogen, they don't whistle Dixie. I say Dixie because we're here in Virginia.

I put that to you in exactly that unreal way. If I can establish with statistical reliability that every time -- or within 90 odd percent -- that organism whistles Dixie we know that it is seeing a substance which we know from animal tests causes cancer in mice and rats, and you can test it out with unknowns by then feeding it to rats and mice, then you have got an empirical test which is, within a certain statistical statement, reliable. It doesn't matter that it's bacterial, Drosophila, or, in the case of the Japanese, the eggs of silk worms. It doesn't matter what it is, and it doesn't matter what the response is, as long as I can tell you what the correlation is and what the mathematical probabilities are.

All that does for you, then, is give you a much quicker way of screening compounds and finding out whether, if you did test them with animals, they would cause cancer in animals. Now that is one feature of what we're doing here, but it's not the whole thing.

The other side of it is that the methodology that's used for whistling Dixie happens to be, in our case, mutations. That has two meanings. One is that it's just plain whistling Dixie. It doesn't matter whether it's mutations or anything else, but there is another significance. People may suffer mutations. You have to worry about that. Therefore, those data have a dual meaning, and the Drosophila work is an effort to take mutation effect and put it into a complex animal where, for example, you have whole chromosomes, which bacteria don't , so that you can begin to get some sense whether those things cause mutations in people, because they may not, even though they cause mutations in bacteria.

In other words, what's confusing is, there is a multiple motivation in these things. One is the purely empirical attempt to save money and time, and the work that I'll describe after lunch is that and nothing else. That's all it is. And it's enough, it works. So that you now can save the money and time.

It also tells you something about some of the compounds. For example, we've discovered that captan is positive in our system, and I can tell you the probability with which that means that if captan is fed to a mouse or rat it will cause cancer.

All hard data are going to be stated in probabilities. There are no absolutes in this thing at all.

And the last thing I want to say is, after this is all done, all you know is that you've got a substance that, with a certain probability, is likely to cause cancer in rats and mice. After that there's a big, big problem of figuring out whether it causes cancer in people, and the good news about that is that I think we're beginning to get a method for going from the one group to the other.

DR. DATTA: Do you think the support we gave you through the chemical program, which is very meager, will be able to summarize for us what type of guidelines on mutagenesis tests that we can give to the registrant to bring in data for us, or do you think that you'll need at least 3-5 years of support from us?

DR. COMMONER: My guess is that within a year it will be possible to turn over to industrial companies a protocol for running the Salmonella tests they can run themselves at about the cost that I gave you, which will enable them to screen any compound or intermediate or pilot plant material. We have now tested the set of knowns, and what's important now is to start testing mixed unknowns and to develop some more methodology for that. There is also a little refinement on the system that has to be done to eliminate a few false negatives.

Once that's done, I think it can be delivered. We have counted 43,000 plates in the last year in our laboratory at a cost of $125,000. That's a hundred compounds, but we're losing money. No, if it weren't for a few things, to play safe I would say a compound will cost you $300 to $500, and you can get through the work, if you want to push it, in 3 days.

I think in a year that can be delivered to any reasonable commercial company. That's for compound, shall we say preparations. For environmental samples, let's say an extract of soil that has been treated, it is going to take somewhat longer, but again, I think after a year and a half's work on our part, we'll have a protocol that will enable you to do the similar analysis, fractionate the compounds, and I'll show you how this afternoon, let's say on an extract of animal fat that's been fed something.

DR. CRANMER: I think Dr. Newell had a comment, but I think I would like to draw on the lead given by the Deputy Administrator last night when he said that scientists sometimes comment on the law as well as lawyers commenting on science, and I believe -- it's been a while since I read it -- but FIFRA, as amended, said something about proving not just the toxic effect of a compound, but a reasonable expectation that that effect will be effected on man or the environment under conditions of use. I believe that's correct, isn't it?

COMMENT: Reasonable adverse effect.

DR. CRANMER: Reasonable adverse effect. So, indeed, I think we all agree with Dr. Commoner that the utility of your first level of evaluation in the systems is great. The utility in making that last analysis may be a difficult road, at least it seems to me it might.

DR. NEWELL: I am attempting to provide you with information and background about utility and reliability of these procedures. There remain 15 to 20 percent inconsistencies of response, and I believe because of this the EPA did not include these in vitro systems in the proposed registration guidelines.

I believe Dr. Commoner will tell us this afternoon how to overcome some of the false negatives, but we still have other problems. Seven months ago, when we were intensively discussing these methodologies with people from various governmental agencies, one of the emphatic comments made by Dr. John Weisberger was, "For God sakes, don't say this is an absolute method at this time. We need at least a year or more for development before people can run with it."

There is a lot of interest in these systems, and it looks like an interesting tool, but much needs to be done with it.

COMMENT: I think this is really the problem, though, in terms of this new situation that's developing where compounds are banned just because they're presumed to be carcinogens, and this I think relates to one or two positive tests, even though there are many negatives. If there are enough data to suggest that it's possibly a carcinogen, that seems to be all that's needed.

QUESTION: What specific example of a compound?

DR. COMMONER: Can I make a comment? I just want to pick up one thing. What you're saying is that some of the in vitro tests work and others don't work. So why do you seize on the one that works and call it a presumptive carcinogen? That's why I said whistling Dixie. If there is one method -- it doesn't matter what it is -- that you can show mathematically correlates, then the fact that others don't work doesn't mean a thing. This is simply an empirical correlation.

Now it means a lot with respect to the theoretical questions and so on, and I think this has to be understood. People say well, this thing works with Salmonella but it doesn't work with yeast. So which one are we going to believe? The question is, what is the mathematical correlation of the Salmonella data to the animal data, and what is the mathematical correlation of the yeast data to the animal data? It turns out that the Salmonella correlations are good, the yeast correlations are bad; therefore you can, if you want to, use the Salmonella data. I don't advise using one with a bad correlation.

Now at the Japanese-American meeting there was a very interesting report given on AF which is a food additive. Two years ago it was discovered to be positive in the Salmonella test, and the people who did it said this is likely to be a carcinogen toward animals and we should take it out of the market. The Government was cautious and said no, let's test it against animals. It was tested against animals, and 2 years later it was found to be positive, and it was taken off the market.

Now what would the Salmonella test do? It saved 2 years and saved money. As I said, it doesn't matter whether the yeast test doesn't work. If you've got one that works with a certain probability, and you want to save 2 years of human exposure, then you use it.

COMMENT: Dr. Commoner, there is one thing that we come across all the time. It's become very difficult on our part, as a regulatory agency. Say, for example, two chemicals have a very similar structure, but one is found to be positive, whereas the other is found to be negative. Now what do you do? Do you study one for 2 years and not study the other for 2 years? Or what do you do? What would you recommend to the company that they should do?

DR. NEWELL: I think it's worth a comment, because Dr. Dale raised a question about the long-term studies. However, let me emphasize again that we're talking about a mutagenic response, and that's why I think in this case a translocation study should be done on captan. In that procedure, you're looking at a potential heritable effect, and you're looking at germinal cells, not somatic cells, and that is the difference, as Dr. Commoner pointed it out. Don't imply that captan is necessarily a carcinogen because of these studies. It may not even be a mutagen.

QUESTION: I'd like to make a direct question to Dr. Newell. This is in regard to the study where you had done the diet incorporation studies for a period of 7 weeks, followed by mating of 8 weeks. This was incorporating the ten compounds in the diet. Did you use TEM intravenously as a positive control in these studies?

DR. NEWELL: Not intravenously. We've used TEM both ways. Early in the game we gave it intraperitoneally and produced a postive response in animals for about five weeks. We now add TEM to the drinking water. We use only a 4-week treatment, a period long enough to affect the cells in the postmeiotic stage. There's no need to treat with TEM for 7 weeks.

QUESTION: Didn't you make the comment that you didn't think this was the way to go, diet incorporation?

DR. NEWELL: No, I said it was the way to go. And that's the way we're testing all of these compounds by incorporating in the diet.

USE OF MUTAGENESIS TEST
TO INDICATE CARCINOGENESIS

Dr. Barry Commoner*

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CRITERIA FOR COMPARISON OF TERATOGENESIS PROTOCOLS

Dr. Herbert J. Schumacher*

In 1966, the FDA and, in 1967, the World Health Organization developed principles for testing of chemicals for teratogenicity. These recommendations still constitute the basic framework of experimental teratology testing even though a great many refinements in testing procedures have been proposed and adopted by numerous investigators.

However, no single test or combination of experimental animal tests yet designed can predict absolutely that a particular environmental agent constitutes a teratogenic risk in man. Therefore, doubts have been cast on the relevance and predictive value of teratogenesis testing, but according to present knowledge, there is no chemical assumed to be teratogenic in man that has not produced malformation in rodents or lagomorphs.

Furthermore, the predictive value of animal tests can be enhanced by paying attention to the following criteria which are essential, but are often overlooked in the outcome of comparative studies designed to safeguard the human population.

During this presentation we will discuss some specific considerations which we feel are important in experimental teratology design and must be accounted for in the interests of standardization, if you will, so that protocols can be adequately compared.

First of all, the state of health and standard care of the animal must be carefully controlled. Animals that are used for teratological studies must be healthy and should be housed under the best possible environmental conditions. A high standard of animal care must prevail. The animal quarters should provide controlled temperature, humidity, adequate light, and protection from noise and other interference since these factors are capable of inducing malformation in one or more species.

Proper caging must be considered; overcrowding, for example, may increase the noxious effects of chemicals that act upon the central nervous system. Since the administration of a chemical may change the food consumption of treated animals, it may

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be necessary to adjust food intake of control animals correspondingly. Results reported in the literature indicate that nutritional stress or imbalance may produce malformation or modified action of other teratogens. Thus diet must be carefully controlled in any teratological study.

For example, the reduction of about 30 percent of food intake in mice produces effects on the teratological outcome of the results. Fifty percent food reduction is needed to produce the same results in rats, and no effect was observed in rabbits. Animal bedding is another factor to be considered, since bedding fumigated with ethylene oxide may interfere with teratological studies, in that vitamins and proteins can be destroyed or transformed by this chemical.

A similar situation may exist with pine bedding or with pesticide residues in bedding or in feed, since the rate of biotransformation of the test material could be changed following alteration of microsomal activity of the experimental animal. Mycotoxins, plant toxins, and naturally occurring steroidal and estrogenic compounds in the feed can also alter the outcome of teratological tests.

Microorganisms and parasitic infections induce teratogenic effects in experimental animals. Furthermore, significant differences in litter size, body weight gain, and water consumption have been reported on distilled water instead of tap water which is available ad libitum.

Type and form of feed also affect the litter size and the pup weight. The time of dosing of animals should be held constant to avoid further variability of the results. The species of choice is also important in teratogenic screening of chemicals. It should have an easily determinable time of fertilization with a high rate of fertility. The present state of knowledge of teratology, the embryology and genetic background, as well as incidence of spontaneous and inducible malformations, should be considered in the species or strains to be chosen.

At least two species, one rodent and one lagomorph, should be used, according to the Agencies, in order to fulfill the general stereotypic requirements. If only such species are used for testing, then age and parity should be known for the following reasons. In polydacious animals, such as mice, hamsters, rats, and rabbits, declining reproductive capacity with increasing age has been demonstrated. Studies indicate that in mice the highest rate of spontaneous anomalies occurs in the first litter and decreases to a very low incidence in the fourth litter.

In rabbits and rats reduced litter size with increasing age was observed, but the incidence of spontaneous malformation was unaffected by maternal age and parity.

Multispecies tests, as proposed by Wilson, should be used. This proposal has the advantage of grading the number of animals used from plentiful in the less expensive rodent species to fewer in the more expensive species. Furthermore, such a procedure would provide much needed multispecies data and the nonrodent species would not have the yolk-sac placenta. Generally the routes chosen for teratological testing should simulate the exposure of man. In the case of oral route, one must determine whether the compound is absorbed by the species in the study. Oral administration should be by standardized gavage or with capsules, since the amount of the compound ingested can be accurately determined and usually insures high levels of absorption.

In some studies administration of the chemical may be in the food or drinking water on the strict condition that the chemical stability and homogeneity of the feed has been ascertained. Food and water consumption has to be determined accurately. The use of intravenous, intramuscular, subcutaneous, and intraperitoneal routes generally is without problems, but interpretation of the results may be difficult when the local tolerance at the site of injection is poor. In inhalation studies, stress, restraining, and/or hypoxia may affect the outcome of results.

Finally, during the course of treatments females should not be subjected to any kind of rough handling, which in itself could cause disturbances of pregnancies as, for example, resorptions or cleft palates.

Treatment periods generally cover three periods in mice and rats. For example, there are 0 to 19, 6 to 15, and 7 to 9 days of pregnancy treatment regimens. The experimental difficulties encountered in routine teratogenic testing include some problems inherent to the action of chemicals. Compounds which destroy the intestinal flora, chemicals with prolonged or intensive pharmacological activity, and substances causing enzyme induction or inhibition which will rapidly harm the health of the mother can only be administered for a short period of time.

It may be useful, or even necessary, to limit the treatment of the pregnant female to a few days, or even a single day, provided that the whole period of organogenesis is covered by using an appropriate number of animal groups. Furthermore, the pharmacokinetic characteristics of the compound in animal species or strains should be taken into account. The selection of adequate doses is important and the following factors must be taken into account: 1) the purity and the physical chemical characteristics of the specific chemicals to be administered; 2) the nature of the pharmacological activity exerted by the compound in the particular species; 3) the dose at which this activity manifests itself; 4) the degree to which man is exposed to the chemical; and 5) pharmacokinetics and metabolism in animal species should be compared with those in man. Such data could be obtained from acute studies, preclinical trials, or by the surveillance of workers in the factories.

There are distinct species, strain, and even interstrain differences in the response of embryos to both suspected and documented teratogenic chemicals. These differences appear to be multifactorial and are related to the fact that teratogenesis is not a single toxic response, but is, rather, the result of a complex interaction between the stage of development at which a compound is administered, the variability time of the compound for placental transfer, its cellular localization within the embryo, and its mechanism of action in situ.

There is, however, one aspect that has not been consistently evaluated in experimental animals which we feel may provide a more meaningful mechanistic interpretation of the results. A combined analysis of placental transfer kinetics and localization within the embryo of either chemical or its metabolites should be undertaken. Only when information from such studies is forthcoming will it be possible to relate both the dose administered to the dam and the dose reaching the embryo to the observed biological effect. It is obvious that when many other parameters, such as plasma half-life, metabolism, protein binding, and tissue distribution in adult animals, are measured, they provide little information to account for the large differences in offspring susceptibility. However, a combined analysis of these parameters, together with placental transfer data, will allow an evaluation of factors which control exposure time and permit the study of the presumed mechanism of action of a chemical or metabolite in situ.

Other factors are the toxicity of the chemicals to the mother animal which will ultimately determine the regimen of treatment. The influence of the carrier substance or the solvent should not seriously influence absorption, distribution, metabolism, or excretion, or alter toxic characteristics or chemical properties of the test compound. The particle size of the test chemical, if it is insoluble in the chosen vehicle, must be sufficiently small and well-defined and its size stated because of the role this parameter plays in absorption.

Factors originating from metabolic interactions between various pesticides, food additives and the variety of environmental chemicals have to be taken into consideration. These effects are due to microsomal activity of the liver, placenta, and/or offspring, which may be stimulated or inhibited. In this way, substances foreign to the body may modify the metabolism of other chemicals that are administered simultaneously.

With this consideration in mind, it is desirable that the highest dose studies should exert some systemic effect in the pregnant female. It is important that the highest dose does not cause excessive embryo lethality, and the fetal loss should not be higher than 50 percent. The lowest dose used should not show any systemic effect in the dam.

Teratogenic effects produced by extremely high doses, compared with human dosage, are probably relevant only when these doses do not impair the well-being of the mother, and if they are not accompanied in a dose-dependent fashion or by other disturbances of the pregnancy.

In order to establish statistically a valid dose-response relationship, generally three to five doses should be chosen. At least two of the treatments scheduled should be utilized in any teratogenic investigation. A series of control animals of equal age should be treated with the vehicle under the same condition as the animals receiving the compound under study. The number of animals per series must be sufficient to enable a statistical evaluation of the results. All doses should be expressed on a milligram/kilogram/day basis. If the compound is given in the diet or drinking water, the dose should then be expressed on a milligram/kilogram/body weight/day basis. Experience has demonstrated that screening studies involving 20 to 40 pregnant rodents per group for each dose, and three to five groups per dose, can provide a good indication of possible effects on the offspring, provided the studies are properly set up and well conducted.

This would include randomized assignment of animals to control and experimental groups and randomized assignment of cage positions on the racks. Careful replication of experimental conditions and the repetition of experiments increase greatly the confidence with which an experiment can be evaluated. For studies in which two or more methods of examination are employed -- e.g., when some litters are delivered by cesarean section and other dams are allowed to rear their offspring for studies on survival, regeneration, or behavior, or when visceral or skeletal examinations are performed separately -- it is advisable to have at least 20 litters for each method of examination.

For larger species, cats, dogs, pigs, sheep, and primates, the number of animals should be as large as one can handle in order to obtain reproducible results and to accumulate much needed background information on such species. Equivocal results should be clarified by further experimentation. The investigator may adjust the dosage and/or dosing period to achieve a more pronounced and recognizable response.

There are different methods for examination of offspring. There is at present no method that allows all of the fetuses to be examined for visceral and skeletal abnormalities. Fetuses should be randomly selected for examination and examined by an observer without prior knowledge of maternal exposure.

In the reviewing of different teratological testing protocols, it becomes apparent that universally acceptable definitions of malformations and definitions of degrees of fetal toxicities are needed. The litter or the fetus can be considered in evaluating teratogenic effects. Both units are of importance. Generally, the litter is the unit for statistical analysis.

For the interpretation of teratological results, the following statistical methods are mainly used. The two-way analysis of variance, the regression analysis, and tests of best fit must be used to ascertain significance. Chi-square test, Fisher's exact test, and the T-test are mainly used in the analysis based on the individual fetuses. Much more work in statistics is needed to maximally evaluate the teratological results and to evaluate risks.

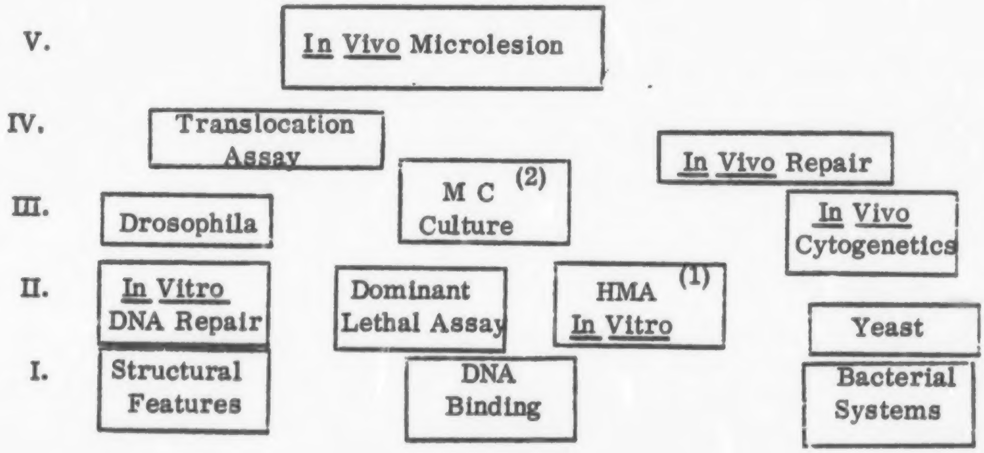
Finally, we suggest that only by standardization of teratogenic testing protocols, by use of the best available and agreed-upon methodology, and by use of well-trained personnel, can we replace the existing confusion in comparing the results of teratogenic research between laboratories and different species of animals. However, standardization should in no way be interpreted as a hindrance or deterrent to the innovative development of new and better methodology.

FEASIBILITY STUDY: DETECTION OF CHEMICAL-INDUCED MUTATION BY ASSAY OF METABOLIC CHARACTERISTICS

Dr. Harvey W. Mohrenweiser*

Monitoring undue exposure to potentially mutagenic agents and thus reducing the incidence of new mutational events is a key to preventing the suspected negative impact of mutations on the health and well-being of the population. In order to prevent the undue exposure, it is essential that systems capable of predicting mutagenic activity be incorporated into toxicological evaluation protocols. Toward this goal, the Division of Mutagenic Research at the National Center for Toxicological Research is attempting to develop an integrated approach to the determination of mutagenic potential by organizing an extensive battery of assays in a pyramidal array; other people may call it the tier approach or various other names. As indicated in Figure 1, this is a theoretical example. There are some obvious exclusions. This was intended just to make sure you would not read anything into the figure that's not there.

Figure 1
Theoretical Pyramidal Array


V.
In Vivo Microlesion
IV.
Translocation Assay
In Vivo Repair
M C (2) Culture
III.
Drosophila
In Vivo Cytogenetics
II.
In Vitro DNA Repair
Dominant Lethal Assay
HMA (1) In Vitro
Yeast
I.
Structural Features
DNA Binding
Bacterial Systems

(1) Host-mediated assay

(2) Mammalian cell culture

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The pyramidal array can be composed of a range of potential test systems with varying degrees of complexity and utility. These will include such things as simple test systems, as we heard Dr. Commoner talk about this afternoon, or some of the things that Dr. Newell talked about this morning. They may be very sensitive to either limited or broad range agents. It will also include more complex systems which may be appropriate for estimating the relative degree of risk associated with normal exposure, such as the Specific Locus Assay, which is the one currently being utilized in mutagenic assay systems.

As you will notice on the lower portion, we have very simple sorts of tests, structural features, DNA binding, and some of the bacterial systems. As you go up one more layer, you observe a more complex system, dominant lethality and the *in vitro* and *in vivo* systems, including the Drosophila system. In no way do I mean to imply that this is an appropriate organization, but it gives you an idea of our thoughts on the organization of more complex systems.

Our thoughts are that this integrated array of test systems should serve as a guide to direct accumulation of the scientific data necessary for valid regulatory decisions. It is again important to emphasize that this is only an example of the tests available and indicative of a possible organization.

It should also be pointed out that such important features as comparative metabolism, pharmacokinetics, dosimetry, and molecular biology data will be necessary, particularly if transitions between various tests and extrapolation from the various tests, particularly at the upper level of the pyramid, are to be meaningful. These latter areas of comparative metabolism, pharmacokinetics, and dosimetry studies particularly are areas in which we are now currently developing active research programs.

We have two programs currently underway, one which I will talk about and the other which Dr. Casciano will talk about. The one I'm going to talk about is a feasibility study of detection of chemically induced mutations by assay of metabolic characteristics. Special emphasis in this area has been placed on the development of *in vivo* mammalian systems which would be useful for determining the relationship between level of exposure and induction of microlesions.

We define microlesions as point mutations, small deletions, other sorts of genetic damage in the mammalian system which are not identifiable by currently utilized microscopic techniques. Currently the major effort is directed toward determining the feasibility of utilizing the techniques employed in the study of inborn errors in metabolism as a method for detecting induced microlesions.

Table 1 is an outline of the approach we are using. Three different technical approaches are being used to detect altered gene products. The first one monitors normal enzyme activity, as well as preliminary kinetic analysis, and heat stability measurements are made on various enzymes from liver, brain, kidney, heart, and red blood cells.

Table 1: Detection of Metabolic Events by Assessment of Metabolic Characteristics

I. Enzyme Activity
    a) Normal activity
    b) Heat stability
    c) Kinetic parameters

II. Electrophoretic Assays
    a) Isoelectric focusing
    b) Isoenzymes
    c) Structural proteins

III. Metabolite Assay
    a) Serum and urine chemistry assay for compounds which are associated with 70 human metabolic errors

The second component is electrophoretic studies of enzyme and nonenzyme proteins These are conducted to look for structural alterations which are not detectable by changes in activity parameters. In the third, the metabolic status of plasma and urine is monitored as an indication of the various storage diseases, metabolic blocks, and other pathway alterations.

At this point we have determined the appropriate assay conditions for apporximately 40 liver and kidney enzymes. The majority of these enzyme analyses are performed on a miniature centrifugal fast analyzer (see Clinical Chemistry 20:1003, 1974 for discussion), which was built for us by Oak Ridge National Labs.

The repeatability of this system when using one microliter of a liver supernatant is such that we have a coefficient variation of about 1.4 to 1.5 percent. If we go to 2 microliters of sample, our coefficient of variation is down to about 0.4 or 0.5 percent. We have one reference cuvette and 16 sample cuvettes, so that in one run we're able to make 16 enzyme assays. Our turn-around time with some of the liver enzymes, particularly those like LDH which are fairly high in activity, is about 4 minutes when we're working at 30 degrees centigrade. The total assay volume for our work is 110 to 140 microliters. With liver we normally work with 1-5 microliters of a one plus six liver homogenate. With tissues like brain, kidney, and heart, we work with a one plus seven or one plus eight and normally work with 3 or 4 microliters this time.

This instrument can be utilized for most enzyme assays which can be coupled to either a product or a substrate within an absorption peak between 290 and 880 millimicron. Approximately 5,000 assays per week can be completed with the current instrumentation.

At this point we have about 40 assays that we are currently able to do. We are also working on new enzyme assays, and we would like to be capable of assaying in the neighborhood of 60 to 70 different enzymes. With the assays being done on various tissues, when we get fully operational, each mouse should be assayed for approximately 115 to 130 different enzymes by direct enzymatic analysis.

Table 1: Detection of Metabolic Events by Assessment of Metabolic Characteristics

I. Enzyme Activity
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II. Electrophoretic Assays
- a) Isoelectric focusing
- b) Isoenzymes
- c) Structural proteins

III. Metabolite Assay

Serum and urine chemistry assay for compounds which are associated with 70 human metabolic errors

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As indicated in Table 1, we are also looking at blood and urine parameters. We now have normal values on approximately 50 different plasma and urine constituents. These components are associated with 70 known human metabolic diseases. We're also currently in the process of developing our electrophoretic studies. It is estimated that with the techniques currently in hand, this approach of enzyme activity measurements, electrophoretic mobility, and metabolite assays will allow us to directly or indirectly examine the gene products for 200 to 300 loci.

Our initial experiment is outlined in Tabla 2. We are using radiation at this time. Obviously, when you're attempting to develop a new method and attempting to standardize your assay conditions, you're best off trying to relate it to the normal established data which are currently in the literature.

Table 2: Treatment and Mating Scheme for Irradiation Experiment

a) F_0 ♂ treated ↓ x F_0 ♀ untreated
b) F_1 ♂ x F_1 ♀ heterozygotes
c) F_2 ↓ also heterozygotes
d) Tissue taken from F_2; urine collected from F_1 for metabolic assays

We are using C57BL/6 mice, and matings were set up for examination of mutations induced in mature sperm as well as stem cell mutations. Two groups of animals (F_0) were irradiated with either 300 or 600 rads. These treated males were then mated to untreated females with production of an F_1 population. This F_1 population would be heterozygous at any loci where mutations occur.

Obviously, we are concerned about heritable events. We mate the F_1s which will give us an F_2 population, and remember, each of these F_1 s is a unique individual, so that even though we do go to an F_2 generation, this F_2 generation will be heterozygous at any loci or most any loci we want to look at.

Remember also that each F_2 individual could be heterozygous at several different loci. Based upon data derived from studying inborn errors of metabolism in humans in excess of 95 percent of a sample of about 200 inborn errors of metabolism where we know the enzyme defects, it is possible to detect enzymatic differences between heterozygotes and either homozygous recessive or homozygous normal individuals.

At this point we have collected urine from approximately 90 F_1 individuals in a very preliminary sort of screening; using basically yes and no answers, we have several which look interesting. We are currently completing the work on quantitative analysis of the urine.

We have looked at five different metabolic errors in mice obtained from Jackson Labs to prove that we can differentiate by determination of enzyme activity within small samples between homozygous normal, homozygous abnormal, and heterozygotes. We've also worked out or are in the process of working out our background data base, using the C57 black mice. Although we are using irradiation, we will in the near future, if and when we get the techniques worked out, make a selection of appropriate chemicals. Obviously, these could be pesticides.

Although most of our activity is directed toward *in vivo* microlesions, as soon as we have the various background features worked out, we will use this system as a means of detecting differences based on both structural and functional relationships. We'll make a direct effort toward quantitating the dose which gets to the germ cells in these C57 black mice.

FEASIBILITY STUDY: THE USE OF DIPLOID CELL STRAINS FROM SPECIFIED MOUSE GENOTYPES FOR USE IN DEVELOPING *IN VITRO* ASSAY FOR MUTAGENIC ACTIVITY, INDUCTION, AND ANALYSIS OF GENE MUTATIONS IN MAMMALIAN CELL LINES

Dr. Daniel A. Casciano*

As Dr. Mohrenweiser has just described, we are attempting to define an integrated approach to mutagenesis. The objective of the second major program in our integrated approach is to develop new and reliable, rapid and economic methods for estimating the potential mutagenicity of environmental agents utilizing *in vitro* somatic cell culture bioassays.

Two approaches are currently being used. We're using specific established cell systems to quantitate the induction of specific gene mutations as a method for screening environmental agents for their potential mutagenic activity, and we are employing recently derived, "functional" mammalian cells to detect and quantitate specific gene mutations. Each of these approaches that I'm going to describe is currently in a developmental stage.

While no available established cell lines appear to be ideally suited for genetic studies of mammalian cell culture systems, several existing lines have proven usable for the isolation of different classes of mutants. The choice of cell line is primarily by requirement of karyotype stability and high plating efficiency. In addition, since mutational events occur at low frequency, any variant or genetic marker must effectively respond to artificial selection to be useful in mutational studies.

The Chinese hamster ovary (CHO) cell line is presently being used as a model system in our laboratory and in others. This cell line has a stable karyotype, short generation time, grows in suspension as well as a monolayer culture, and possesses a high plating efficiency. In addition, CHO cells have already been successfully used in isolating drug-resistant and auxotrophic mutants. The genetic marker to be selected for is resistance to 6-thioguanine. This selective system has the advantage

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that the gene conveying this drug resistance is X-linked, so that only one gene copy is present per cell. Utility of this selective system allows one to determine whether the variant isolated is a phenotypic or genotypic variant by assaying the gene product, hypoxanthine guanine phosphoribosyl transferase (HGPRT).

In somatic cell culture it is at present possible to obtain dose-response relationships for mutants which are fairly reproducible. However, we are still a long way from determining exact mutation frequencies, which are important for estimation of genetic risk, and from obtaining exact dose-responsiveness, which are necessary for fundamental research on the process of mutation induction.

Recently a sensitive and rapid system utilizing a specific gene mutation affecting the HGPRT locus in CHO cells has been developed at the Oak Ridge National Laboratories. This system, which measures 6-thioguanine resistance, has been employed to quantitate various physical and chemical mutagens and carcinogens. Mutation can be quantitated at mutagen doses, which produces no appreciable cell death, and linear dose relationship has been obtained. We are presently modifying this system to test the dose-responsiveness of a variety of known and potential chemical mutagens which include direct alkylating agents, suspected metabolites of promutagens, and promutagens transformed in appropriate activation systems. Studies are also in progress to quantitate mutation at an autosomal locus, which codes for adenine phosphoribosyl transferase. These systems should provide a means to quantitate specific locus mutations, either at X- or autosomal-linked genes.

In addition to utilizing the CHO cell line as a model system, established cell cultures from a variety of mammalian species, including man, will be adapted for mutagenesis testing. Success in this endeavor would then be followed by attempts to correlate the dose-response in various mammalian cell cultures to specific mutagens with dose-response in human cell cultures. This approach may aid in overcoming the problem of extrapolation to man of results obtained with animal systems.

The second approach that we are using in the somatic system involves use of recently derived "functional" epithelial mammalian cells to detect and quantitate specific gene mutations. We recently have been attempting to derive "functional" liver, kidney, and lung cell cultures originating from the mouse and other mammalian species. At present we are emphasizing isolation of "functional" liver cells. We've used a variety of tissue preparation methods, and we have had success using the Bauscher and Schaeffer technique published in the spring of 1974 in In Vitro. Using 3-day and 9-day-old mouse liver as starting material, we have been able to obtain a proliferating low passage epithelial-like culture, retaining morphology of the cells originally plated in sufficient quantity for liquid nitrogen storage.

We are attempting to characterize these cells as to their true origin. The criteria for functionality we are applying are both morphological and biochemical. The morphological criteria include those shown in Table 1. We are using both operational definitions utilized in cell culture methodology and histological definitions. Table 2 depicts some of the biochemical characters we are using to identify the hepatic cell.

Table 1: "Functional" Mouse Liver Cells

Criteria for Functionality

A. Morphology and Growth Characteristics:

1. Liver parenchymal morphology
2. Limited life span
3. Contact inhibition of growth
4. Unable to grow in agar, serum-free medium or in suspension
5. Do not form tumors when injected into host

Table 2: "Functional" Mouse Liver Cells

B. Biochemical Characteristics:

1. Retention of mixed function oxidases: e.g., aryl hydrocarbon hydroxylase
2. Glycogen synthesis and storage
3. Tryptophan to nicotinamide adenine Dinucleotide biosynthetic enzymes
4. Phenyl alanine hydroxylase activity
5. Hormone receptor sites

The cultures we have isolated from 3-day-old mice are now in the 30th passage, which represents around 60 generations, and are retaining epithelial-like morphology. At present we can identify at least two epithelial cell types, one of which is the so-called clear cell defined by Grisham, whose liver function is unknown. This cell type is presently being utilized in several laboratories for carcinogenesis bioassay. The other epithelial cell that is present in the culture is tentatively being identified as a liver hepatocyte based on its morphological characteristics. We are attempting to clone these two cell types and have recently enriched for each morphology.

We have done some preliminary characterization of the mixed culture, and the results are as follows: The cultures retain epithelial characteristics until the 30th passage. They also display contact inhibition of growth. After the 25th passage, which represents around 50 to 55 generations, the culture is apparently senescing and displaying a limited lifespan. Glycogen is being synthesized and stored up to the fifth passage; this was identified phytochemically using the para-aminosalicylic (PAS) stain; and constitutive aryl hydrocarbon hydroxylase activity is being retained to at least the 18th passage. In addition, of those hepatic enzymes we have analyzed, the cells are displaying an enzyme profile characteristic of the 3-day-old mouse. We are presently deriving cultures from 9-day-old mice whose enzyme profiles are more nearly like that of the adult, and we are having relative success with this.

The functions we are most interested in are the mixed function oxidase systems, which will allow us to use these cultures to analyze mutagenicity of known and potential chemical mutagens requiring metabolic activation. This may allow us to overcome the limitations of presently available established somatic cell systems used in mutagenesis testing.

In addition, we are anticipating tying in with the carcinogenesis programs at the National Center for Toxicological Research, using accepted somatic cell carcinogenesis endpoints. Additionally, we are developing a program to study DNA repair synthesis in these cells when exposed acutely and chronically to known mutagens and carcinogens, and we hope to correlate the effect of various doses of mutagens with DNA repair and mutation frequency. Hopefully, once these systems are developed, we can apply the epithelial cells to toxicological evaluations of a variety of chemicals.

EPIDEMIOLOGY OF PESTICIDES: CANCER MORTALITY AND PESTICIDE USAGE IN THE UNITED STATES

Dr. William F. Durham*

This is going to be fairly brief because it is just an outline of a contract which we have quite recently -- as a matter of fact, June 27 -- negotiated with the School of Public Health at the University of North Carolina at Chapel Hill. I hope that next year Dr. Carl Shy, who is the principal investigator for this contract, will be able to be with us and discuss the actual results.

This contract is designed to investigate the possibility of correlation between cancer mortality and pesticide usage in the United States. Basically what the contract is set up to do is conduct an epidemiologic study to determine whether or not a geographic association can be demonstrated between United States counties of high pesticide usage and counties in which excess cancer mortalities have occurred after appropriate adjustments are made for other known epidemiologic determinants of cancer mortality, such as age composition of the group, sex, race, socioeconomic level, and rural-urban land use patterns.

More specifically, the study will investigate whether counties with high usage of DDT in the 1945-1960 period show any excess in mortality from cancers of the liver, gastrointestinal system, or hematopoietic system or total cancers, minus lung cancer. One of the specific things that Dr. Shy will be doing is to tabulate the cancer mortality for all causes and for specific relevant causes, adjusted by age, for each county in selected agricultural regions of the United States, including as a minimum the Southeast and Southwest, where high pesticide usage has been prevalent.

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Then to correlate with that, Dr. Shy will obtain the pesticide usage by counties for this same 1945-1960 period, and what he will use for this will be data available from the United States Census of Agriculture for the specific counties where these data are available. It may very well be that to get certain portions of this information, it will be necessary to actually go to the records of the county agricultural extension agent and find out what was being recommended during these particular years for pesticide usage on the major crops which are grown in these counties.

Initial analysis will focus on the association of specific classes of pesticide usage — herbicides, insecticides, and fungicides -- with total cancer, cancer of the respiratory system, cancer of the bladder, cancer of the biliary passages and liver, and cancers of the hematopoietic system. Multiple regression analysis, cluster analysis, and factorial analyses will be utilized in addition to simple categorical analyses of discrete variables.

Considering the possibility of the 5-, 10-, 15-, or 28-year time lags between first exposure and death from cancer related to that exposure, the cancer mortality for each sex and race group categorized according to socioeconomic and urban-rural composition as above will be compared in counties with high DDT usage versus counties with low DDT usage.

As I said, this contract was just set up on the 27th of June, so this is just an outline of what is planned.

EFFECTS OF CACODYLIC ACID ON THE PRENATAL DEVELOPMENT OF RATS AND MICE*

Dr. Neil Chernoff**

Cacodylic acid (dimethylarsinic acid) is an arsenic-containing pesticide. It is used for post-emergence weed control, crown kill of trees, and defoliation of cotton. It is also used for the control of bark beetles.

To date, there have been no published data on the possible effects of cacodylic acid on reproduction or teratogenesis. It is known that intravenous injections of sodium arsenate in hamsters (1,2), intraperitoneal injections of sodium arsenate in rats (3), and intraperitoneal injections of sodium arsenate (4), or sodium arsenite (5) in mice produce a variety of fetal malformations when these compounds are administered during the period of organogenesis. The dietary administration of 100 ppm of arsenic trioxide was shown to have no effects on reproduction or neonatal growth rate in rats (6). A case of possible arsenic-induced neonatal death in a human has also been reported (7).

The experiments reported in this paper were carried out to determine the possible fetal effects in mice and rats of an organic form of arsenic in the environment.

Materials and Methods

Random-bred albino mice (CD-1 stock) and rats (CD stock) were used (8). Day 1 of pregnancy was recorded upon demonstration of sperm in the vaginal smear. Animals were in rooms maintained at constant temperature (22-26°C) with controlled lighting (12 h. light) and were fed commercial lab chow and water ad libitum.

Cacodylic acid (9) was administered by gastric intubation during the period of organogenesis on days 7-16 of gestation. Maternal weight on day 6 was used for calculation of doses. The compound was administered as a solution in distilled water

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*Co-author is Ellen H. Rogers

**Experimental Biology Division, Neurobiology Branch, EPA with intubation volumes of 0.1 ml in mice and 0.2 ml in rats. The doses administered were 200,400, and 600 mg/kg/day in the mouse, and 40, 50, 60, 75, and 100 mg/kg/day in the rat. Distilled water controls were run concurrently.

Mice and rats were killed on days 18 and 21 of gestation, respectively. The uteruses were removed and weighed. Maternal weight changes during gestation were calculated as the weight of the intact animal at the time of sacrifice less the weight of the removed gravid uterus, from which the day 6 weight was subtracted. Live fetuses were weighed and the litter divided into either Bouin's solution or 65 percent ethyl alcohol. All fetuses were examined for external defects. After 3 to 5 days, necropsy was performed on those fetuses fixed in Bouin's solution. Fetuses in ethyl alcohol were subsequently cleared in 1 percent KOH and stained with alizarin red S to facilitate skeletal examination.

Calculations were based on the litter as the experimental unit, and standard deviation therefore represent the variation of litter means. Comparisons between treatment and control groups were made using the Mann-Whitney "U" test (10). Dose-response relationships were tested with Jonckheere's test (10).

Results

The data from the rat studies are summarized in Table 1. The two highest doses, 100 and 75 mg/kg/day, resulted in maternal mortality of 80 and 33 percent, respectively. There was little or no maternal mortality at the other dose levels. The only significant alterations in maternal weight occurred at the 100 and 75 mg/kg/day doses. No changes in liver/body weight ratios were noted at any dose levels.

The administration of cacodylic acid resulted in significant ($P<0.001$) dose-related reductions in fetal weight, sternal ossification centers, and number of caudal vertebral ossification centers. Fetuses weighed an average of 3.41 g at the 75-mg dose as compared to 4.10 g in control litters. Sternal and caudal ossification centers were reduced from averages of 5.6 and 4.6, respectively, in control litters, to 4.1 and 3.8 in litters within the 75-mg dose treatment group. There was increased fetal mortality at all dose levels greater than 40 mg/kg/day.

Micrognathia (small jaw) was noted in litters of treated animals at all dose levels. The micrognathia ranged from slight to severe shortening of the mandible. Isolated examples of small lungs whose lobes did not extend ventral to the heart as in controls were found in the 50, 60, and 75 mg/kg/day doses. A common alteration in treated fetuses was aberrant rugae on the palates. In control fetuses the large anterior rugae met in opposition at the midline ridge of the palate. In many of the treated litters at all dose levels the pattern of these large anterior rugae was not symmetrical and the individual pairs of rugae did not lie in apposition.

The data on the mouse studies are summarized in Table 2. Significant dose-related reductions in maternal weight gain ($P<0.01$) and liver/body weight ratios ($P<0.001$) were noted during the course of the study. Maternal mortality (7 percent) occurred in animals receiving the high dose of 600 mg/kg/day.

Significant dose-related reductions in fetal weight ($P<0.001$), sternal ossification centers ($P<0.01$), and the number of ossified caudal vertebrae ($P<0.001$) were noted. The average fetal weight in litters within the 600-mg dose group was 0.93 g, as compared to 1.12 g in the controls. Sternal and caudal ossification centers were reduced from an average of 5.7 and 5.0, respectively, in control litters to 5.1 and 3.2 in litters of animals treated with 600 mg/kg/day. A slight increase in fetal mortality was found at all dose levels.

Litters containing one or more fetuses with cleft palates were found at the 400- and 600-mg dose levels. The palatine shelves were in the horizontal position in all affected fetuses.

Discussion and Summary

The administration of cacodylic acid to CD rats during the period of organogenesis resulted in fetal toxicity, as indicated by dose-related reductions in weight and in the degree of ossification of both the sternum and caudal vertebrae. Micrognathia was noted at all dose levels. A number of grossly small lungs were evident at the 50-, 60-, and 75-mg dose levels. This anomaly is difficult to identify strictly on a morphological basis, principally because many of the fetuses themselves manifested significant reductions in size. Experiments utilizing a quantifiable index such as a lung/body weight ratio are currently ongoing. The significance of the aberrant palatal rugae is not known. It should be noted that no cases of cleft palate were found during the course of the rat studies, even in litters with one or more fetuses exhibiting aberrant rugae.

In the mouse, the administration of cacodylic acid at doses of 200-600 mg/kg/day during the period of organogenesis resulted in a spectrum of fetal toxicity similar to that found in the rat, but its teratogenic response differed markedly. Dose-related reductions in the average fetal weight and degree of ossification were noted. Unlike the rat, however, the only anomaly which was seen in the mouse was cleft palate. This defect occurred only after the administration of 400 and 600 mg/kg/day dose levels which also resulted in some degree of maternal toxicity, as evidenced by a reduction in maternal weight gain during pregnancy.

Observations on the effects of perinatal cacodylic acid administration are continuing, as are studies on the possible postnatal sequelae to prenatal exposure and studies involving both technical grade and the pure compound.

References

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6. Kojima, H.: Studies on development pharmacology of arsenite. 2. Effects of arsenite on pregnancy, nutrition and hard tissue. Folia Pharmacol. Japon. 70: 136-149 (1974) (Abstract)

7. Lugo, G., Cassady, G., and Palmisano, R.: Acute maternal arsenate intoxication with neonatal death. A. J. Dis. Child. 117: 328-330 (1969)

8. Animals were obtained from the Charles River Breeding Laboratories, Wilmington, Massachusetts.

9. The cacodylic acid which was used in these experiments was purchased from ICN Pharmaceuticals. Analysis by the Ansul Chemical Co. indicates that the sample had approximately 5% NaCL, 1.7% methane arsenic acid, 0.2% arsenoris ions, 0.7% arsenic ions, and 2.0% unidentified organic compounds.

10. Hollander, M., and Wolfe, D. A.: "Nonparametric Statistical Methods." New York, John Wiley and Sons, 1973, pp. 71, 120.

Table 1: Prenatal Effects of Cacodylic Acid in the Rat

Data	Dose Group (mg/kg/day)					
	Control	40	50	60	75	100
Conception						
No. inseminated	28	15	32	11	18	21
No. died	0	0	1	0	6^{e}	17^{e}
% mortality	0	0	3	0	33	80
No. pregnant (term)	23	11	26	9	8	2
Maternal observations						
Av weight gain (g)	68.4 ± 20.1	78.4 ± 17.9	63.1 ± 26.7	77.8 ± 19.3	56.0 ± 15.8^{c}	-9.8^{c}
Av liver/body weight	4.8 ± 0.4	5.1 ± 0.3	4.8 ± 0.3	5.0 ± 0.3	4.9 ± 0.5	5.2
Fetal observations						
Av implants	10.0 ± 3.0	10.2 ± 3.2	9.7 ± 2.1	10.0 ± 2.5	9.8 ± 1.5	8.5
Av mortality (%)	4	4	21^{c}	31^{d}	18^{c}	82^{d}
Av weight (g)	4.10 ± 0.29	3.79 ± 0.31^{d}	3.50 ± 0.48^{e}	3.28 ± 0.61^{e}	3.41 ± 0.52^{e}	2.40^{e}
Av no. sternal ossification centers	5.6 ± 0.5	5.5 ± 0.5	5.1 ± 1.1^{c}	4.3 ± 2.0^{c}	4.1 ± 1.8^{c}	2.5^{e}
Av no. caudal ossification centers	4.6 ± 0.4	4.2 ± 0.9^{c}	3.9 ± 0.9^{d}	3.4 ± 1.5^{d}	3.8 ± 1.0^{b}	1.0^{c}
Small lungs^{a}	0	0	3(5)	2(2)	1(1)	0
Micrognathia^{a}	0	2(2)^{b}	7(12)^{b}	3(7)^{c}	4(15)^{d}	1(1)
Aberrant rugae^{a}	1 (4)	10(31)^{e}	8(28)^{e,f}	8(26)^{e}	-	-

a Data given as no. of litters (no. of fetuses).

b Comparing these groups with controls produces $P < 0.10$.

c Statistically different from controls, $P < 0.05$.

d Statistically different from controls, $P < 0.01$.

e Statistically different from controls, $P < 0.001$.

f Only 9 litters examined for this anomaly.

Table 2: Prenatal Effects of Cacodylic Acid in the Mouse

Data	Dose Group (mg/kg/day)			
	Control	200	400	600
Conception				
No. inseminated	53	46	56	55
No. died	0	1	1	4
% mortality	0	2	2	7
No. pregnant (term)	38	27	38	35
Maternal observations				
Av weight gain (g)	5.0 ± 2.5	5.2 ± 1.8	3.8 ± 2.4[a]	3.5 ± 2.3[b]
Av liver/body weight	7.3 ± 0.6	7.4 ± 0.5	6.8 ± 0.7[b]	6.9 ± 0.7[b]
Fetal observations				
Av implants	12.0 ± 2.2	12.0 ± 2.3	11.4 ± 2.9	11.9 ± 2.5
Av mortality (%)	7	13	12	15
Av weight (g)	1.12 ± 0.19	1.06 ± 0.19	0.92 ± 0.20[c]	0.93 ± 0.19[c]
Av no. sternal ossification centers	5.7 ± 0.6	5.6 ± 0.6	4.7 ± 1.7[b]	5.1 ± 1.4[b]
Av no. caudal ossification centers	5.0 ± 1.9	4.4 ± 2.0	2.7 ± 1.8[b]	3.2 ± 2.0[a]
Cleft palate, no. of litters (no. of fetuses)	0(0)	0(0)	3(4)	9(18)[e]

a Statistically different from controls, $P < 0.05$.
b Statistically different from controls, $P < 0.01$.
c Statistically different from controls, $P < 0.001$.

DR. KENAGA: Has this compound been studied in any long-term feeding studies, like reproduction studies or any other long-term studies before?

DR. CHERNOFF: Not to my knowledge.

DR. KENAGA: Is this the first time that the teratogenicity has been known for this compound?

DR. CHERNOFF: To my knowledge, yes, this is the first teratogenic study of this nature using this sort of protocol. I don't know if three-generation studies have been done and perhaps not published.

MR. BURNAM: I don't think we have any previous teratology studies on cacodylic acid or reproduction studies either. It was tested in the bionetics cancer study. It came out negative there. But I don't think there are any other long-term studies with it.

ASSESSMENT OF SUBTLE AND DELAYED EFFECTS OF SUBSTITUTE CHEMICALS

Dr. Daniel A. Spyker*

I will first present some brief background on how we came to propose this program to the EPA, describe our rationale, and then describe the methods by which we propose to integrate the conventional teratological approach with maturational, behavioral, endocrinological, and immunological evaluation of experimental animals over their entire lifespans. I will then describe what we hope to have in the way of data to present at next year's conference.

Background

In 1967, we began studying a widely used and very effective fungicide, methylmercury. In a teratological assessment of this compound we documented a variety of effects, all of which were dependent on dose, stage of development when exposed, and strain of animal. None of the mothers of affected offspring showed any signs of toxicity. These responses for the fungicide, methylmercury, were present at a dosage which caused no gross detectable abnormalities in the mothers. Further, in doses which were not teratogenic and in animals which during their early development showed no signs of gross abnormalities, we found their behavior to be abnormal in the open field. In the evaluation of 20 treated and control animals, we found differences in 4 of the 8 parameters measured, including center latency, number of defecations, number of urinations, and in backing behavior. As these animals grew older, such gross neuromuscular deficits became evident as ataxia, loss of righting ability, and what we might loosely refer to as premature aging. We also found conjunctivitis to develop more frequently in treated animals. Cultures were positive for pneumococcal bacteria, and we conducted a preliminary screen on immunologic status. An evaluation of T-cell function using sheep red blood cells showed no differences between treated and control animals, but Brucella challenges of the B-cell system, although not different in the young animals, did show significant differences in response in the mature animals. These and other data have caused us to feel that the organism is significantly more vulnerable during development than it is at any other time. It seems likely that certain specific systems (for example, the immunological and, certainly, the CNS) may be at greatest risk.

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Program Objectives

We propose an integrated study to detect deficits that are not readily apparent and/or are initially latent but are expressed later in life. In addition to testing for deficits that may be subtle and delayed, we will further enhance the sensitivity of our evaluation by evaluating the organism at risk, i.e., when exposed during development, and within that organism evaluating the systems most susceptible to insult, i.e., the CNS, endocrine, immune systems, and looking at these systems over the animal's entire lifespan.

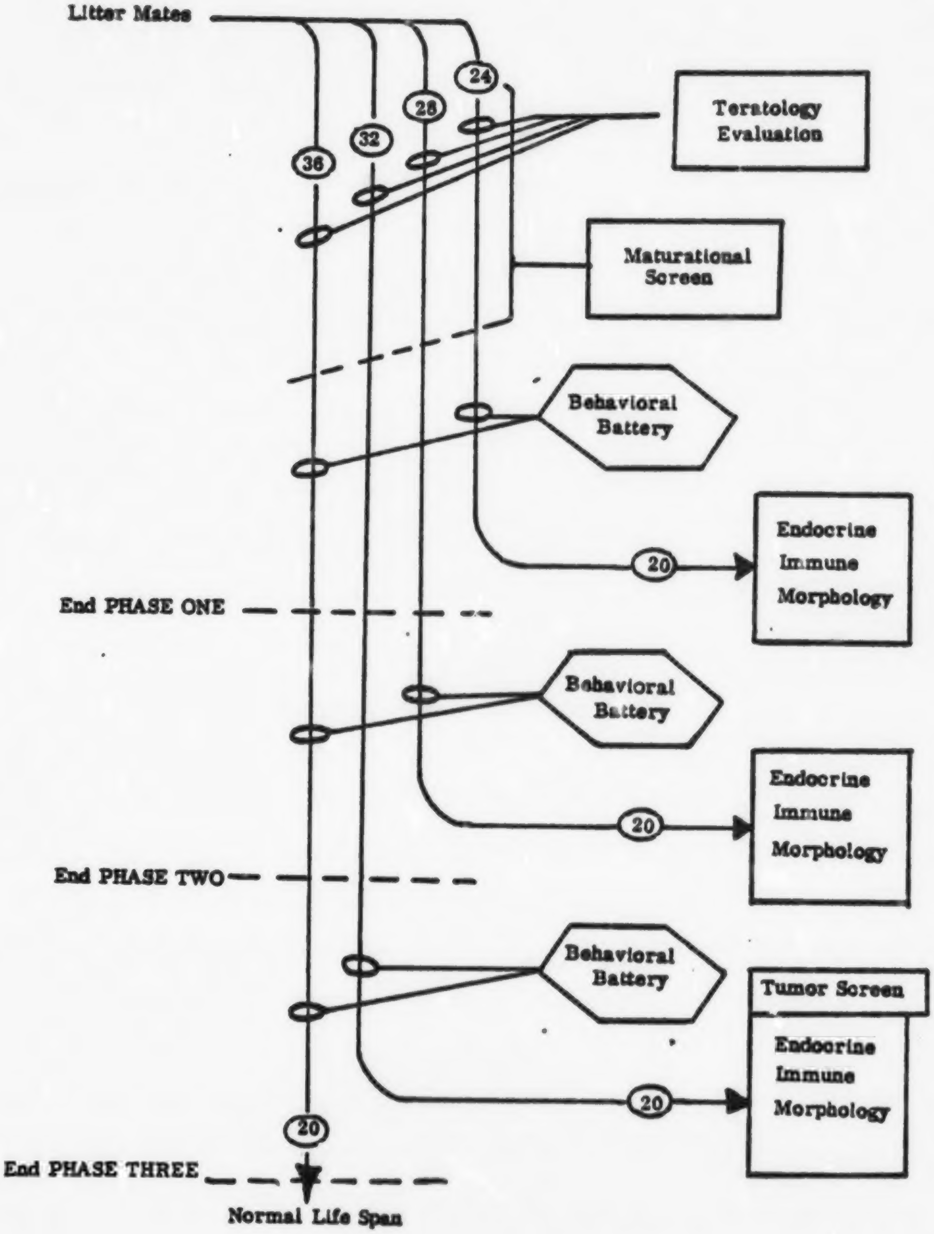
We will use an inbred strain of mice for this study, 129/SvSl. Pesticides tested include chlordane, heptachlor, parathion, carbofuran, methoxychlor, and Diazinon. All six compounds will be administered orally and, in addition, the animals will be exposed to chlordane and parathion by inhalation. The oral route will be used to simulate the human situation. Dosage will be based on levels monitored in the environment, and duration of exposure will be based on knowledge of the compounds' kinetics such that the fetal environment approximates the steady state situation for the dosages chosen. For example, the chlorinated hydrocarbons are strongly liquid bound, bioaccumulate, and require longer exposure than the two organic phosphorus compounds, We will initially determine an acute LD_{50} for non-pregnant adults to compare the toxic data for our animals with that reported for each compound in other test animals. Our approach is summarized in Figure 1 which is a schematic flow of animals through the lifespan evaluation. This will be replicated for each test compound and twice for control groups. The numbers represent the number of test animals in each of the 4 tracks, e.g., 36 animals will be evaluated on 3 separate occasions in behavioral batteries and allowed to live out their normal lifespans. Litter mates will be assigned to these four tracks as far as possible to minimize the intratrack variation.

Teratologic Examination

Although we expect to be using a dosage which will not cause overt defects, we can efficaciously collect teratologic data on these test animals. These data will include

Figure 1
Schematic Flow of Animals Through the Three-Year Evaluation

This basic design is replicated for each of the eight (i.e., two control and six pesticide) experimental groups. PHASE ONE is designed to generate enough tested animals to permit subsequent evaluation over their lifespan and correlation of results with early measures.


Litter Mates
24
28
32
36
Teratology Evaluation
Maturational Screen
Behavioral Battery
20
Endocrine Immune Morphology
End PHASE ONE
Behavioral Battery
20
Endocrine Immune Morphology
End PHASE TWO
Behavioral Battery
Tumor Screen
20
Endocrine Immune Morphology
20
End PHASE THREE
Normal Life Span

physical characteristics, fetal or neonatal lethality, congenital malformation, growth retardation, and overt functional deficits.

Maturational Screen

We will attempt to assess maternal-neonatal interaction by assessing maternal retrieving response, nursing, and cannibalization. Offsprings' physical development will be monitored by recording the age at eye opening, development of a righting reflex, and development of forelimb placing. Testes descent and vaginal opening will also be monitored for correlation with endocrinological evaluation. Birth weight and weaning weight or weight gain is an important index of general health. Sensory development will be assessed by observing each offspring's performance on visual cliff, auditory orienting, and olfactory aversion tasks. Strength and coordination will be tested using rotarod, plank walk, bar holding, and rod climb.

Behavioral Battery

Three major tasks have been chosen for this evaluation. These individual tests or tasks are designed to stress an array of different functional systems so as to maximize the likelihood of detecting behavioral deficits if they are indeed present. Differences, if uncovered, will be subsequently evaluated in more discriminative testing to isolate the specific system or process involved. The animal's behavior in a novel environment is a test which assesses emotionality, activity, locomotion, and exploration. This is done by placing the mouse on a brightly lit, large, open surface and observing its activity. This technique is commonly known as open field testing.

Learning, memory, and cognition will be tested in a modified Lashley III maze. We will use a four baffle maze which control animals typically learn to run flawlessly in approximately 75 trials. After acquisition, the difficulty of this task may be altered by simply imposing a time limit on execution of the maze. Time discrimination may be assessed by only reinforcing certain running times longer than the animal's previous scores. For example, if the animal can complete the maze in 30 seconds, his reward can be withheld unless he now executes in between 40 and 50 seconds.

A stressful neuromuscular task will be imposed on these animals in the form of swimming over a specified time in ice water. This test provides assessment of the animal's coordination, equilibrium (vestibular system), and response to stress. This task will be recorded on videotape for objective evaluation. Immediately upon completion of the task, blood will be drawn for an immunoassay of plasma-corticosterone.

Endocrinological Evaluation

No single measurement is available to provide assessment of the entire pituitary-adrenal axis; therefore, three tests will be employed in this initial screen to identify sites of interest for further study. One, plasma-corticosterone concentrations will be determined by radioimmunoassay, both at rest and after application of the standardized swimming stress. These differences will serve as a crude measure of overall responsiveness of the axis. Two, whole adrenal glands will be incubated with added ACTH under standardized in vitro conditions, and steroid production will be assessed as an indicator of adrenal secretory capacity. Three, liver tissue will be homogenized and incubated in vitro with added corticosterone. A-ring and side-chain metabolism can thus be assessed, providing not only a measure of hepatic function per se, but also an estimate of the biological clearance rate of corticosterone.

Gonadal function testing will consist of determination of the age of vaginal opening or testes descent, as was previously outlined. Female cyclicity will be checked at appropriate intervals by vaginal cytology. Gonadal weights will be obtained and tissues fixed for subsequent immunologic evaluation. Plasma samples will be stored for subsequent measurement of testosterone or estradiol.

Immunologic Assessment

We propose to evaluate both the general and specific immune status of the mice exposed to each pesticide. The general immune status will be assessed through measurement of the circulating immunoglobulins IgG, IgG_2, IgA, and IgM. This will be determined by radial immunodiffusion or specific precipitant techniques, as appropriate. Single cell suspension from each spleen will be evaluated to determine numbers of lymphocytes and/or macrophages. Lymphocyte subpopulations of those carrying surface immunoglobulins, i.e., B-cells, and those possessing receptors for erythrocytes, i.e., T-cells, will also be determined.

Specific immune status assessment will initially focus on the previously described decrease in antibody response to the Brucella abortus. Challenge via the flagelin molecule in polarized form permits assessment of T-independent response, whereas the monomer challenge will permit assessment of T-dependent response. Additionally, we will study serum protein antigens (using sheep red blood cells and bacterial polysaccharides). We will evaluate the kinetics of antibody response using the plaque-forming cell technique, and the cellular immune response by the graft vs. host reaction using a spleen weight bioassay technique.

Morphologic Evaluation

If CNS dysfunction is detected among the animals to be sacrificed during the first phase, three brains will be examined for neuropathology under the light microscope. If no CNS deficits are detected, the brains will be fixed and stored for subsequent examination, as indicated. Likewise, if altered gonadal indices are detected, then representative ovaries and testes will be examined for histopathology. Figure 2 summarizes the integrated Phase I effort and evaluation of each pesticide.

Data Acquisition and Analysis

As previously described, data collection will begin at birth but animals are not uniquely identifiable until one week of age when toe tattoo is possible without altering maternal-offspring interaction. At that time each animal will be randomly assigned a unique mouse number which will thereafter be its sole identification. A cross-reference which will include its treatment history will be maintained, but will be used only in assigning animals for individual tests or in analysis of results. The principal instrument of data collection will be individually prepared, encounter form type data sheets. These forms represent the only necessary hard-copy record in the data collection process.

For each encounter form a special-purpose interactive data entry program for the Biotronics computer system will be used. This program requests the operator to enter each piece of datum in a format which matches the encounter form, checks each entry at the point for reasonableness, and writes the data onto a magnetic disk file after all entries have been approved by the operator. This data file will be subsequently manually checked against the encounter form and the file will be rewritten and regrouped by treatment and sex according to the master key. The resulting file thus becomes one of the master data files. This procedure is summarized in

Figure 2
Integrated Team Effort

The six-way integrated assessment of animals exposed during development is diagrammed below. Broken lines represent planned and potential interaction among the several discipline specialities and animal groups involved in this study.

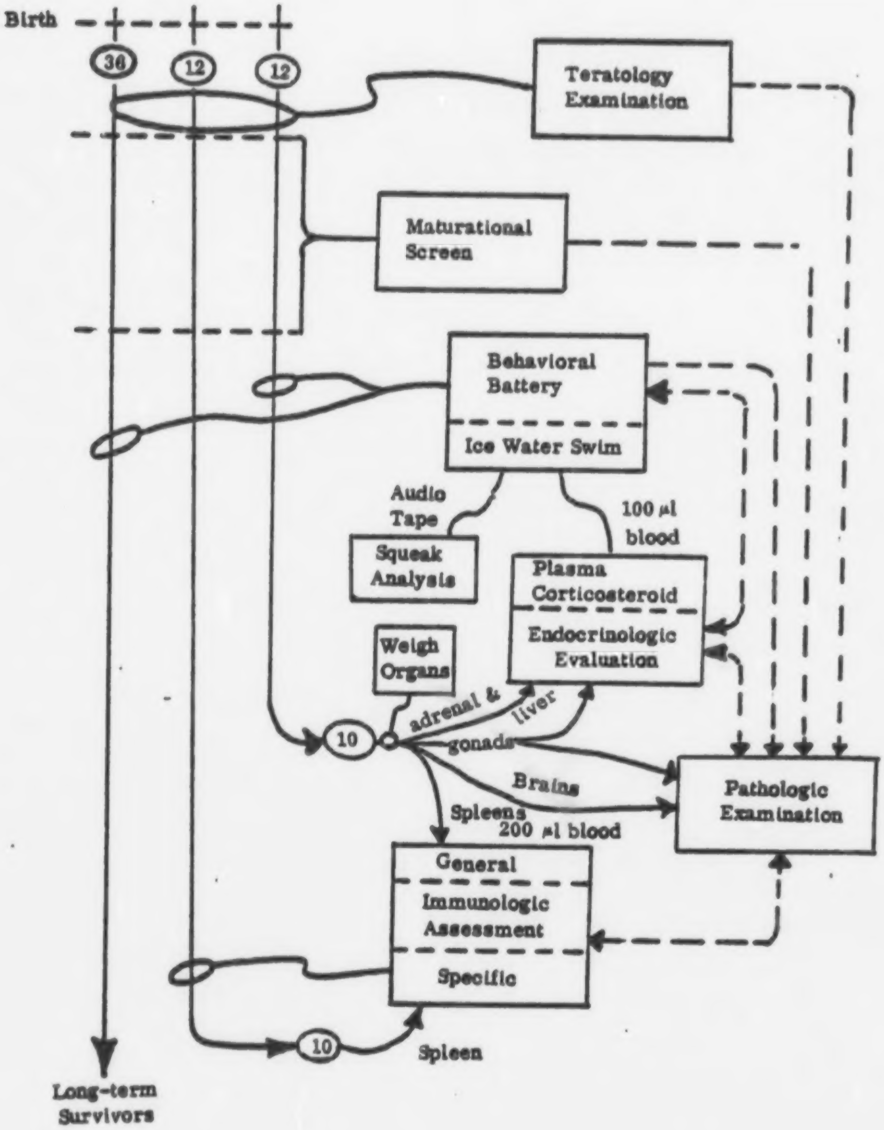

Birth
36
12
12
Teratology Examination
Maturational Screen
Behavioral Battery
Ice Water Swim
Audio Tape
100 µl blood
Squeak Analysis
Plasma Corticosteroid
Endocrinologic Evaluation
Weigh Organs
10
adrenal &
liver
gonads
Brains
Spleens
200 µl blood
Pathologic Examination
General
Immunologic Assessment
Specific
10
Spleen
Long-term Survivors

Figure 3. We may hypothesize that any one of the measured parameters is unaffected by the treatment, the "null hypothesis." Replication of the t-test for each parameter is not legitimate, but, of equal importance, it neglects information inherent in the simultaneous nature of the observations. In the age-old conflict between the means and the variances, we propose to tip the balance in favor of the means by use of one or more multivariable statistical techniques. For each set of data, for example the open field observations, we will apply multivaried discriminate analysis. This will provide not only a single measure of the discriminating power of the test, but will also provide a rank ordering of the contributions of each individual measure to this discrimination.

Product

As a result of this contract, we hope to provide:

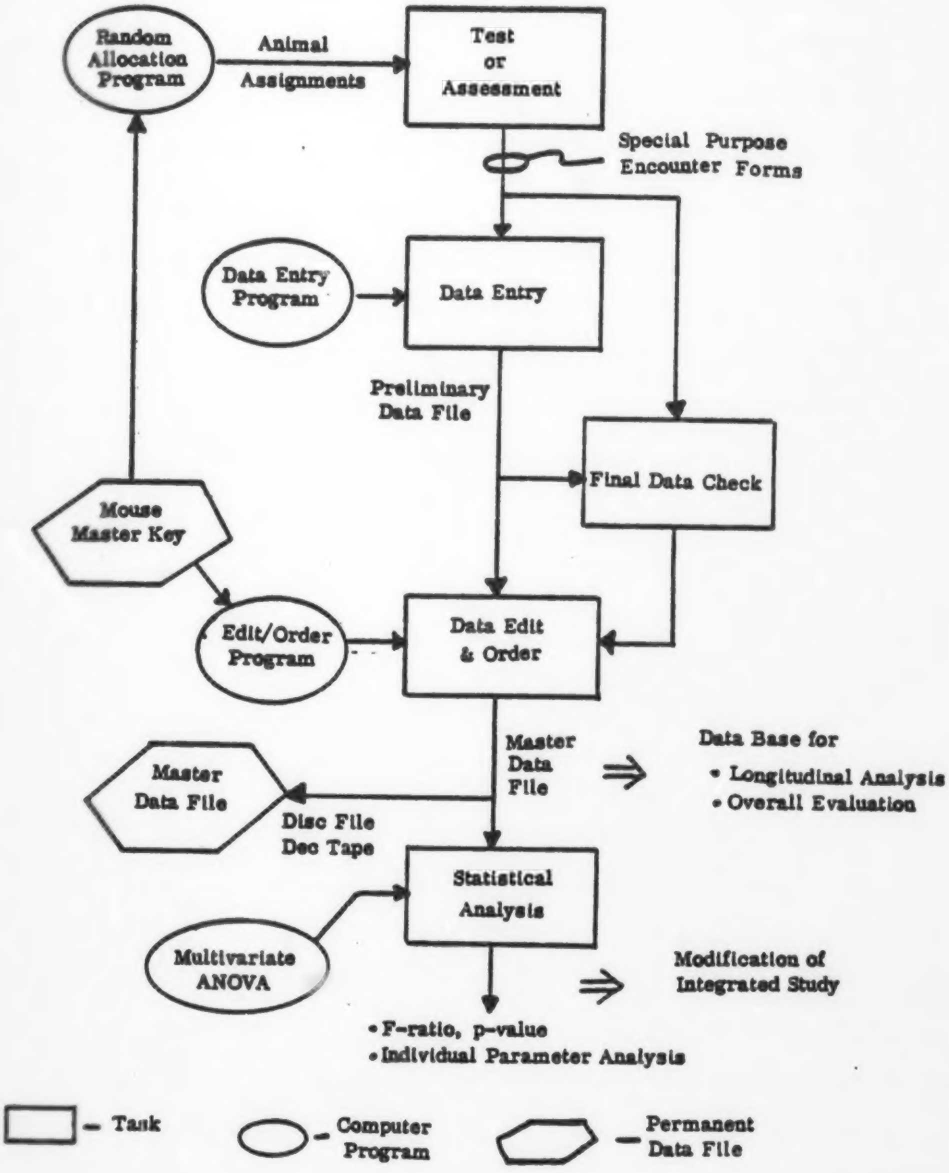
1) Data base, consisting of integrated data on teratology, maturation, behavior, endocrinology, immunology, and pathology for each compound tested.

2) Conclusions, regarding the quantitative effect of each compound on the developing organisms over the total lifespan.

3) In addition, this approach will evolve a particular methodology or protocol for each compound tested. That is, it will pinpoint the particular system or target organ for each compound and permit subsequent efficacious testing of similar compounds. It may similarly suggest testing procedures for humans exposed to these or similar compounds.

Figure 3
Data Acquisition and Analysis

Schematic of data flow, to be replicated for each test or assessment, i.e., for each task.


Random Allocation Program
Animal Assignments
Test or Assessment
Special Purpose Encounter Forms
Data Entry Program
Data Entry
Preliminary Data File
Final Data Check
Mouse Master Key
Edit/Order Program
Data Edit & Order
Master Data File
Data Base for
• Longitudinal Analysis
• Overall Evaluation
Master Data File
Disc File
Dec Tape
Statistical Analysis
Multivariate ANOVA
Modification of Integrated Study
• F-ratio, p-value
• Individual Parameter Analysis
– Task
– Computer Program
– Permanent Data File

QUESTION: Dr. Spyker, I was interested in one technical aspect. Maybe your wife could answer this better, but in the swim test, which is the test for performance under stress and the like, you emphasized the aspect of ice cold water instead of just swimming itself. Have you ever run any comparative tests putting an animal in, you might say, ambient water versus ice water? Is there a difference? Do you get greater stimulation, or what's the reason for using ice water? It's a detail, but I think it's rather interesting.

DR. SPYKER: I agree. We have done both ambient water and ice water swims. The data reported in Science were obtained in ambient water. I don't have any large numbers and large means of small variances of P values to report to you. I'm sure, as your question implied, you believe that there's more stress involved in swimming in ice cold water. In fact, mice can swim for hours in ambient water, whereas survival time is under a minute in ice water.

DR. CURLEY: Did you do any histopathology on the harderian glands, the salivary glands, or did you see any swelling in the necks of these animals that might be associated with a viral infection and not with treatment with the compound in question? Or, I may phrase it another way. Viral infections are endemic in some colonies of rats. Was this the problem with these animals that you treated with, I guess it was methylmercury? The question is precautionary.

DR. SPYKER: The question is well founded, I'm sure, and my answer will be a hedge. We did not do the histopathology or the gross pathology that you question. Our defense is the best available under the circumstances. That is that our data implied a higher incidence of infection in the treated group than in the control group, which was housed, you know, within a few feet. The cultures of the eyes were positive for pneumococcus predominantly. That does in no way preclude a viral infection. But it was very tidy, or perhaps, better, reassuring that the defect we did discover was in the B-cell system, which we normally think of as being more responsive to bacterial, pneumococcal type infections, rather than viral. So that's the only response I have. That's a good recommendation. We'll take that seriously next year.

DR. BARON: I couldn't quite make out on the flow chart whether some of your behavioral studies were going to encompass weanling animals, young animals. My first impression was that your tests started at the age of 12 months or 24 months, and seemed to proceed thereafter. Are we going to avoid behavioral testing on young animals, especially since we know that with some organophosphorous compounds, some of the maximal inhibition is usually noted within the first 6 to 8 weeks after the animals, weanling animals, are put on test? This would show the maximal effect on these young animals, which then thereafter proceeds to equalize itself and the animals lose their sensitivity to immunosuppression. Is your test limited to the older animals after 12 and 24 months?

DR. SPYKER: Our experimental design really involves four groups of animals. One group will receive the complete behavioral battery shortly after weaning while two other groups will not be tested until years two and three. The final group will be tested repeatedly, beginning at weaning and again at intervals throughout the lifespan.